Biological degradation of substrate mixtures composed of phenol, benzoate and acetate by *Burkholderia cepacia* G4

Biologischer Abbau von Substratgemischen aus Phenol, Benzoat und Acetat durch *Burkholderia cepacia* G4

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von
Moustafa El-Sayed Abd El-Hameid Shalaby
aus Kafr El-Sheikh, Ägypten

1. Referent:	**Prof. Dr. W.-D. Deckwer**
2. Referent:	**Prof. Dr. Ing. D. C. Hempel**
eingereicht am:	**06. Februar 2003**
mündliche Prüfung (Disputation) am:	**07. Mai 2003**
Druckjahr:	**2003**

ACKNOWLEDGEMENT

The work described here was carried out between October, 1999, and December, 2002, in the Biochemical Engineering Division of the GBF (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) in partial fulfilment of the requirements for the Degree of Ph. D.

I wish to express my deepest gratitude to my supervisor Prof. Dr. W.-D. Deckwer, head of the Biochemical Engineering Division, for suggesting the topic of this thesis as well as for his professional guidance and constructive criticism.

My kindly thanks are also for Prof. Dr. Ing. D. C. Hempel for agreeing to act as a co-referee. Deep thanks and appreciation to Prof. Dr. K. Buchholz for agreeing to act as a chairman of the examiners commission.

I am also greatly indebted to Dr. V. Hecht, whose valuable advices, excellent suggestions and permanent motivating support were of inestimable value. Sincere thanks are also to him for language correction of this work.

I wish to express my sincere gratitude to Prof. Dr. K. N. Timmis for providing the bacterial strain used throughout this work. My thanks are also due to Dr. D. Pieper for his guidance for analysis of 2-HMS.

For the friendship, helpful comments, professional advices and nice working environment, I wish to thank Mr. L. Bischoff. I would also like to thank Mr. S. Rühe for his excellent cooperation to determine the HPLC-analytical parameters. For the technical assistance I wish also to thank Mr. J. Nothnagel.

My deepest thanks are also extended to Prof. Dr. M. E. K. Ibrahim, professor and head of the Microbiology Division, Kafr El-Sheikh Faculty of Agriculture, Tanta University, Egypt for his professional advices and permanent support during his supervision in Egypt.

I would like to take this opportunity to express my deeply gratitude to my parents, my wife, my children Nourhan and Ahmed for their love and encouragement during preparation of this work.

I wish also to extend my sincere gratitude to the government of Egypt for the financial support.

Finally, I am grateful to everybody who helped and encouraged me during this study.

LIST OF CONTENT

SYMBOLS AND ABBREVIATIONS

1. INTRODUCTION 1
1. 1. Review of literature 5
1. 1. 1. Cultivation of the single substrates 5
1. 1. 1. 1. Biological degradation of phenol 5
1. 1. 1. 2. Biological degradation of benzoate 8
1. 1. 1. 3. Biological degradation of acetate 10
1. 1. 2. Cultivation of the substrate mixtures 12
1. 2. Aim of the work 17

2. MATERIALS AND METHODS 19
2. 1. Microorganism 19
2. 2. Culture media 19
2. 3. Cultivation system 20
2. 3. 1. Cultivation in shake flask 20
2. 3. 2. Cultivation in bioreactor 20
2. 4. Construction and periphery of the reactor 21
2. 4. 1. Reactor 21
2. 4. 2. Bioprocess control system 23
2. 4. 3. Cultivations 23
2. 5. Analytical methods 23
2. 5. 1. Determination of biomass 23
2. 5. 2. HPLC-analytical parameters 24
2. 5. 3. Analysis of 2-hydroxymuconic semialdehyde (2-HMS) 25
2. 5. 4. Effluent gas 25
2. 6. Computational methods 26

3. THEORETICAL BASIS 27
3. 1. Mass balances 27
3. 2. Kinetics 28
3. 3. Stoichiometry 30
3. 4. Calculation of oxygen and carbon dioxide transfer rates 31
3. 5. Special cultivation techniques 31
3. 5. 1. Wash-out cultivation 31
3. 5. 2. A-stat or substrate ramp cultivation 32
3. 6. Carbon balance (carbon recovery) 33

4. RESULTS AND DISCUSSION.....35
4. 1. Substrate biodegradation under chemostat conditions.....35
4. 1. 1. Chemostat cultivation of the single substrates.....35
4. 1. 2. Chemostat cultivation of the ternary mixture of substrates.....43
4. 2. Substrate biodegradation under dynamic conditions.....49
4. 2. 1. Cultivation of single substrates.....50
4. 2. 1. 1. Biological degradation of phenol.....50
4. 2. 1. 1. 1. Batch cultivation.....50
4. 2. 1. 1. 2. Wash-out cultivation.....58
4. 2. 1. 1. 3. A-stat cultivation.....60
4. 2. 1. 2. Assessment of the time delay for the exhaust gas in the reactor 63
4. 2. 1. 3. Biological degradation of benzoate.....65
4. 2. 1. 3. 1. Batch cultivation.....65
4. 2. 1. 3. 2. Wash-out cultivation.....70
4. 2. 1. 3. 3. Modelling of benzoate cultivation.....73
4. 2. 1. 4. Biological degradation of acetate.....78
4. 2. 1. 4. 1. Batch cultivation.....78
4. 2. 1. 4. 2. Wash-out cultivation.....82
4. 2. 1. 5. Conclusion of the single substrate cultivations.....87
4. 2. 2. Cultivation of substrate mixtures.....89
4. 2. 2. 1. Phenol-acetate.....89
4. 2. 2. 1. 1. Batch cultivation.....89
4. 2. 2. 2. Phenol-benzoate.....96
4. 2. 2. 2. 1. Batch cultivation.....96
4. 2. 2. 2. 2. A-stat cultivation.....102
4. 2. 2. 3. Phenol-benzoate-acetate.....106
4. 2. 2. 3. 1. Batch cultivation.....106
4. 2. 2. 3. 2. A-stat cultivation.....113

5. SUMMARY.....121

6. ZUSAMMENFASSUNG.....124

7. REFERENCES.....128

SYMBOLS AND ABBREVIATIONS

a	acceleration factor	$[h^{-2}]$
$a_{C\,CO2}$	mass fraction of carbon in CO_2	$[g\,g^{-1}]$
$a_{C\,i}$	mass fraction of carbon in compound i	$[g\,g^{-1}]$
$a_{C\,S}$	mass fraction of carbon in substrate	$[g\,g^{-1}]$
$a_{C\,X}$	mass fraction of carbon in biomass	$[g\,g^{-1}]$
B.	*Burkholderia cepacia* G4	
C_i	concentration of compound i	$[g\,L^{-1}]$
$C_{i\,0}$	concentration of compound i in the feed	$[g\,L^{-1}]$
CoA	coenzyme A	
C_{rec}	carbon recovery	$[g\,g^{-1}]$
C_S	substrate concentration	$[g\,L^{-1}]$
$C_{S\,0}$	substrate initial concentration	$[g\,L^{-1}]$
C_V	substrate feed concentration	$[g\,L^{-1}]$
C_X	biomass concentration	$[g\,L^{-1}]$
$C_{X\,0}$	biomass feed concentration	$[g\,L^{-1}]$
CO_2	carbon dioxide concentration (exhaust gas)	[%]
CPR	carbon dioxide production rate	$[g\,L^{-1}\,h^{-1}]$
CTR	carbon transfer rate	$[g\,L^{-1}\,h^{-1}]$
D	dilution rate	$[h^{-1}]$
$D_{crit.}$	critical dilution rate	$[h^{-1}]$
DO_2	dissolved oxygen concentration	[%]
F_G	air flow	$[L\,h^{-1}]$
F_L	convective liquid flow	$[L\,h^{-1}]$
K_S	substrate affinity constant	$[g\,L^{-1}]$
K_i	inhibition constant	$[g\,L^{-1}]$
m_{CO2}	maintenance coefficient for carbon dioxide	$[g\,g^{-1}\,h^{-1}]$
m_j	maintenance coefficient for compound j	$[g\,g^{-1}\,h^{-1}]$
m_{O2}	maintenance coefficient for oxygen	$[g\,g^{-1}\,h^{-1}]$
m_S	maintenance coefficient for substrate	$[g\,g^{-1}\,h^{-1}]$
M_{CO2}	molecular weight for carbon dioxide	$[g\,mol^{-1}]$
M_{O2}	molecular weight for oxygen	$[g\,mol^{-1}]$
OTR	oxygen transfer rate	$[g\,L^{-1}\,h^{-1}]$
OUR	oxygen uptake rate	$[g\,L^{-1}\,h^{-1}]$
P.	*Pseudomonas*	
q^{mt}	vector of gas/liquid mass transfer rate	$[g\,L^{-1}\,h^{-1}]$
q^r	vector of biological reaction rate	$[g\,L^{-1}\,h^{-1}]$
r	specific rate	$[g\,g^{-1}\,h^{-1}]$

r_{CO2}	specific carbon dioxide production rate	[g g^{-1} h^{-1}]
r_i	specific rate for compound i	[g g^{-1} h^{-1}]
r_{O2}	specific oxygen uptake rate	[g g^{-1} h^{-1}]
r_S	specific substrate consumption rate	[g g^{-1} h^{-1}]
$r_{S\,max}$	maximum specific substrate consumption rate	[g g^{-1} h^{-1}]
STP	standar temperature and pressure	
TCA	tricarboxylic acid cycle	
V_L	reactor liquid volume (working volume)	[L]
V_M	volume of one mole of ideal gas at STP	[L mol^{-1}]
X_{CO2}^{in}	molar fraction of carbon dioxide in inlet gas	[mol mol^{-1}]
X_{CO2}^{out}	molar fraction of carbon dioxide in exhaust gas	[mol mol^{-1}]
X_{O2}^{in}	molar fraction of oxygen in inlet gas	[mol mol^{-1}]
X_{O2}^{out}	molar fraction of oxygen in exhaust gas	[mol mol^{-1}]
Y	yield coefficient	[g g^{-1}]
$Y_{CO2/S}$	carbon dioxide to substrate yield coefficient	[g g^{-1}]
$Y_{X/CO2}$	biomass to carbon dioxide yield coefficient	[g g^{-1}]
$Y_{X/j}$	biomass to compound j yield coefficient	[g g^{-1}]
$Y_{X/O2}$	biomass to oxygen yield coefficient	[g g^{-1}]
$Y_{X/S}$	biomass to substrate yield coefficient	[g g^{-1}]
$Y_{x/j/TG}$	true growth yield coefficient of compound j	[g g^{-1}]

Greek and Mathematic Symbols

μ	specific growth rate	[h^{-1}]
μ_{max}	maximum specific growth rate	[h^{-1}]
τ	hydrodynamic residence time	[h]

1. INTRODUCTION

During the last few decades, an array of foreign compounds to biological systems called "xenobiotics" has been introduced into the environment. Due to their foreignness, these compounds can cause deleterious effects on living systems and many accumulate in the environment. In different ecosystems, the environmental pollutants are generally found and strongly affected the agricultural, industrial and commercial activities in most countries in the world. The agricultural and industrial wastewaters contain relatively high concentrations of the organic pollutants. These pollutants are usually toxic, mutagenic, or carcinogenic, and may be accumulated or magnified by the biota. Chemically, the environmental pollutants belong to several categories such as: phenols, aldehydes, sterols, ethers, acids, esters, aromatic hydrocarbons, ... etc. (Paxeus et al. 1992).

The need to remove these contaminations has led to the development of new technologies that emphasize the detoxification and destruction of the pollutants rather than the conventional approach of disposal. The key to the assessment of the fate of organic chemicals in the environment is a realistic evaluation of their susceptibility to mineralization (the conversion to carbon dioxide, water and various inorganic forms). Most of these compounds can be used as carbon and energy sources by the microorganisms. Biodegradation means the biological transformation of an organic pollutant to another form (Grady, 1985).

The microbial community in the soil or in a wastewater reactor is, therefore, confronted with a substrate mixture composed of toxic and non-toxic compounds. In addition to the presence of a capable organism, environmental conditions must be suitable for the enzymatic catalysed reactions to proceed at a significant rate. On the other hand, the microbial community varies in its composition according to changes in the feed and the physical-chemical conditions (pH, T, ... etc.). Normally, the concentration of the toxic compounds is much lower than those of the non toxic substances. Regarding the performance of a microbial wastewater treatment system, the basic questions are therefore:

i) Will toxic (inhibitory) compounds be degraded in the presence of non toxic compounds, and if, to what degree?
ii) What is the maximum loading or flow through the reactor at conditions of complete degradation of the toxic compounds?

The high complexity of the system reveals that is not directly suited to optimization methods based on reaction kinetic principles. However, by studying the degradation kinetics of various compounds and microorganisms, knowledge about the kinetic properties of different biological systems can be gathered and used to understand more complex systems in a step by step manner.

The effect of various substrates on the microbial degradation of organic pollutants has been the subject of a number of studies (Bauer and Capone, 1985, Hofmann and Kruger, 1985, LaPat-Polasko et al. 1984, Müller et al. 1996, Rast et al. 1979, Rubin and Alexander, 1983, Schmidt and Alexander, 1985, Shimp and Pfaender, 1985, Singleton, 1994, Smith, 1990, and Wang et al. 1985).

The principles of microbial growth kinetics, which is the relationship between specific growth rate and substrate concentration, were developed mainly between 1940 and 1970. They are based (for non inhibitory compounds) on a limitation of the growth rate by the substrate concentration as expressed in the famous Monod equation (Monod, 1949),

$$\mu = \mu_{max} \; C_S / (K_S + C_S) \qquad [1.\,1]$$

and on a linear relationship between the specific growth rate and the specific substrate conversion rate (Pirt, 1965):

$$\mu = Y_{X/S} \; r_S - Y_{X/S} \; m_S \qquad [1.\,2]$$

It should be noted, however, that equation [1. 2] was derived and is only valid for chemostat operation. The four parameters of the two equations, the maximum specific growth rate μ_{max}, the substrate affinity constant, K_S, the true growth yield coefficient, $Y_{X/S}$, and the maintenance coefficient, m_S, were usually regarded as constant. Since the 70's important work has been published regarding the growth kinetics on inhibitory substrates. The mathematical formulations were mainly deducted from enzyme kinetics and are mainly based on the Monod-equation, so for example the Andrews-equation (Andrews, 1968) for substrate inhibited growth which is equal to the well known Haldane-equation (Haldane, 1930) for enzymes:

$$\mu = \mu_{max} \; C_S / (K_S + C_S + C_S^2 / K_i) \qquad [1.\,3]$$

The usual way to identify kinetic parameters in a bioreactor is based on different cultivation methods. Inhibition constants can only be estimated under conditions of excess substrate, for example in a batch cultivation. On the other hand, batch cultivations are not very well suited to identify substrate affinities, because the substrate affinity does not influences the course of a batch cultivation very much. Substrate affinities can be estimated from steady states near to the maximum dilution rate or better from a slowly but steadily increase of the dilution rate across the washout point (A-stat) (Paalme et al. 1995 & 1997). Yield and maintenance coefficients are usually estimated from steady states. An interesting new cultivation technique was proposed by Schröder et al. (1997). The authors used the multiplicity of steady states of substrate inhibited growth in a chemostat to operate the bioreactor in controlled unstable steady states in the inhibitory branch. This cultivation technique enables the acquisition of inhibition coefficients out of a (quasi) steady state and combines the properties of a classical chemostat and a batch cultivation.

An observation, known since the 1970's and still unsolved, is the often nonexistent transferability of kinetic data derived from continuous cultivations to batch cultivations and vice versa. In this regard, the influence of the "history of the culture" (Kovárová-Kovar and Egli, 1998, Senn et al. 1994, and Sokoł, 1987) is often stated. Also, published kinetic parameters for batch cultivations carried out with the same substrate and the same microorganism showed an amount of variation which can not be explained by data scatter. This aspect is highlighted in the newer literature and the constancy of the kinetic parameters is questioned (Kovárová-Kovar and Egli, 1998). Who also reported that a change of the physiological state of the culture may lead to a change of the kinetic parameters. Grady et al. (1996) already introduced the "intrinsic" and "extrinsic" properties of kinetic parameters. The intrinsic properties are influenced only by the nature of the substrate, the microorganism, and the physical-chemical properties of the environment. They are considered as reproducible. The extrinsic properties are influenced by the current state of the growth or cultivation conditions, respectively.

A review is given by Kovárová-Kovar and Egli (1998). It is suggested that the microorganisms may adapt themselves to the current environmental conditions by altering their kinetic parameters. Senn et al. (1994), summarized that the ability of microorganisms to adapt to restricted availability of food is depending on the growth conditions of a microorganism. At substrate limiting conditions, for example, a microorganism will try to reduce its substrate affinity as far as possible by sacrificing its maximum specific growth rate to gain an advantage in competition with other microorganisms. At excess substrate conditions, on the other hand, the maximum specific growth rate is the most important parameter, whereas the affinity can be higher with no loss in competiting power. In a $\mu_{max} = f(K_S)$ plot, data derived from different culture conditions should therefore not culminate in a specific point but be located in region limited by the intrinsic values of the parameters. The observation that smaller affinity values for certain substrates were measured in substrate mixtures compared with single substrate systems can also be explained by this theory (Kovárová-Kovar and Egli, 1998). However, for an detailed investigation of this phenomenon, reliable and voluminous data sets, derived under different cultivation conditions, are necessary.

The general goal of this work is to investigate the ability of a microorganism to mineralize toxic compounds in the presence of non toxic substrates. Special emphasis will be given to the kinetics of degradation and growth and the variability or non variability of the kinetic and stoichiometric parameters. The biological model system will be composed of the microorganism *Burkholderia cepacia* G4 and the substrates phenol, benzoate and acetate. Phenol is a toxic compound which inhibits its own conversion even at low concentrations (substrate inhibition). Benzoate is metabolized via the same catabolic route with exception of the first step, but is considerably less toxic or inhibitory. Acetate is considered to be a non toxic substrate, and it is a compound of the cells intermediate pathway, so a special catabolic sequence is not needed.

1. 1. Review of literature

Burkholderia (*Pseudomonas*) *cepacia* G4 has already been used for degradation of phenol in the Environmental Biochemical Engineering Research Group at GBF, the most interesting work being the controlling of unstable steady states in continuous culture by Schröder et al. (1997).

Aromatic compounds are widely found in the environment as components of plant materials and as pollutants from anthropogenic sources. Their degradation by bacteria has been studied in order to understand the complex biochemistry involved and to obtain kinetic models that may assist in the bioremediation of such compounds (Bouwer and Zehnder, 1993 and Williams and Sayer, 1994).

Performance of microbial degradation of toxic organic pollutants or xenobiotics, under conditions similar to those of the natural habitat of the organism and especially for use in a bioreactor, is mainly influenced by the ability to maintain high degradation rates and to mineralize the toxic substrates completely. The effect of various substrates on the microbial degradation of organic pollutants has been the subject of a number of studies (Bauer et al. 1985, Hofmann and Kruger, 1985, LaPat-Polasko et al. 1984, Müller et al. 1997, Rast et al. 1979, Rubin and Alexander, 1983, Schmidt and Alexander, 1985, Shimp and Pfaender, 1985, Singleton, 1994, Smith, 1990, and Wang et al. 1985).

The degradation of aromatic compounds proceeds through well-known catabolic pathways. These compounds are converted to one of dihydroxyaromatic substrates, such as: catechol, gentisate and protocatechuate (Ornston and Yeh, 1982, and Ribbons and Eaton, 1982). These intermediates further undergo ring fission to yield metabolites, such as pyruvic acid, acetic acid, succinic acid and acetyl-CoA, for the krebs cycle (Johnson and Stanier, 1971). Of the three dihydoxyaromatic intermediates, the most frequently encountered metabolite before ring cleavage is catechol. Once catechol is formed, it can be degraded through either the *meta* or the *ortho* ring cleavage pathways. Chloro-substituted and unsubstituted catechols are mainly degraded through the *ortho* (ß-ketoadipate) cleavage, whereas alkylsubstituted catechols degraded through the *meta* (α-ketoadipate) cleavage pathways starting with the ring cleavage enzymes: pyrochatechase (catechol 1,2- dioxygenase) or metapyrochatechase (catechol 2,3-dioxygenase), yielding 2-hydroxymuconic semialdehyde (2HMS) and cis, cis-muconate, respectively (Cain and Farr, 1968, Commandeur and Parsons, 1990, Davies and Evans, 1964, De-Lipthay et al. 1999, Farrell and Quilty, 1999, Feist and Hegeman, 1969a,, Nozaki et al. 1963, Sala-Trepat and Evans, 1971, Stanier and Ornston, 1973 , and Timmis et al.1988).

Many microorganisms are capable to utilize the different environmental pollutants under aerobic and/or anerobic conditions. Pseudomonads have demonstrated the ability to mineralize a wide variety of aromatic compounds (Brilon et al. 1981, Kilpi et al. 1983, Knackmuss and Helwig, 1978, LaPat-Polasko et al. 1984, Loh and Wang, 1998, Ribbons, 1970, Sheela and Pai, 1983, and Zeyer and Kearney, 1984).

Burkholderia cepacia G4 (formerly *Pseudomonas cepacia* G4) is one of the most effective bacterial strains able to use a wide range of the environmental pollutants (Folsom et al. 1990).

The bioremediation of polluted groundwater and toxic waste sites requires that bacteria come into close physical contact with pollutants. This can be accomplished by chemotaxis (Parales et al. 2000).

Of the aromatic moiety degraders, *Burkholderia cepacia* G4 is one of the microorganisms described to have high degradation rates of trichloroethylene (TCE) (Folsom et al. 1990). Folsom and Chapman (1991) have investigated the degradation of TCE by using phenol as growth and energy substrate in a model bioreactor. Cometabolic conversion of trichloroethylene occurs also by using the same bacterial strain in the presence of either toluene monooxygenase (Nelson et al. 1987 and Shields et al. 1989 & 1991) or phenol (Hecht et al. 1994) as co-substrates and inducers. On the other hand, a wide range of aromatic compounds can be completely utilized by strain G4 with high consumption rates, for example, toluene, o-cresol, m-cresol, or phenol for TCE degradation activity (Nelson et al. 1986 & 1987).

1. 1. 1. Cultivation of the single substrates

1. 1. 1. 1. Biological degradation of phenol

Phenol and its derivatives are common constituents of wastewater originating from many industries including pharmaceutical, petroleum and coal refining. Additionally, phenol is a common pollutant due to its presence in the effluents of the agricultural activities. Phenol is a toxic and hazardous substance even at low concentrations (Hill and Robinson, 1975, and Li and Humphrey, 1989) and efficient treatment methods are necessary to reduce phenol concentration in wastewater to acceptable levels. Of the several methods available for treatment of phenol, biological treatment is especially attractive because it has the potential to almost degrade phenol completely with producing innocuous end products and minimum secondary waste generation (Goudar et al. 2000).

Biological degradation of phenol has been extensively investigated, and several studies have shown that phenol can be aerobically degraded by a wide variety of microorganisms, including pure bacterial cultures such as: *Acinetobacter calcoaceticus* (Paller et al. 1995), *Alcaligenes eutrophus* (Hughes et al. 1984 and Léonard and Lindley, 1998), *Bacillus stearothermophilus* (Buswell, 1975), *Burkholderia cepacia* G4 (Folsom et al. 1990, Müller, 1994, Schröder et al. 1997 and Solomon et al. 1994), *Nocardia sp*. (Rizzuti et al. 1979), *Nocardioides sp*. (Cho et al. 2000), *Pseudomonas pickettii* (Fava et al. 1995), *Pseudomonas putida* (Bettmann and Rehm,1984, Feist and Hegeman, 1969a, Götz and Reuss, 1997, Hill and Robinson, 1975, Hinteregger et al. 1992, Sokół, 1987 & 1988b, Yang and Humphrey, 1975, ... etc.), *Pseudomonas resinovorans* (Dikshitulu et al. 1993), *Ralstonia eutropha* (Léonard et al. 1999) and *Rhodococcus sp*. (Straube, 1987).

On the other hand, some yeasts are also capable to breakdown of phenol such as: *Candida spp*. (Hofmann and Krüger, 1985, Krug and Straube, 1986 and Neujahr et al. 1974), *Fusarium sp*. (Anselmo et al. 1985) and *Trichosporon cutaneum* (Gaal and Neujahr, 1979, Neujahr and Gaal, 1973 and Spånning and Neujahr, 1987). Different reviews deal with degradation of phenol by mixed bacterial cultures (Farrell and Quilty, 1999, Mörsen and Rehm, 1990, and Pawlowsky and Howell, 1973).

Information about the experimental conditions is necessary for operation of the biological treatment system required to degrade phenol. Kotturi et al. (1991) investigated a model low temperature system at 10˚C to mineralize phenol by *Pseudomonas putida* Q5. On the other hand, most of phenol cultivations were carried out at 30˚C and pH was kept at 6.8 ± 0.05 by using different strains of *Pseudomonas putida* (Hinteregger et al. 1992, and Sokół, 1987). Whereas, a range from 23 to 28˚C was a suitable temperature to degrade phenol by *Burkholderia cepacia* G4 (Bremer, 1994, Folsom and Chapman, 1991, Saéz and Rittmann, 1993).

Catechol is the main intermediate resulting from metabolism of phenol by different microbial strains. As for the aromatic compounds, phenol can be cleaved by *meta* or *ortho* routes. During degradation of phenol, *meta* cleavage characterized by catechol 2,3-dioxygenase was induced by different strains such as: *Alcaligenes eutrophus* (Léonard and Lindley, 1998), *Pseudomonas cepacia* (Ghadi and Songodkar, 1994 and Nelson et al. 1987), *P. pickettii* (Kukor and Olsen, 1991) and *P. putida* (Feist and Hegeman, 1969a, and Hill and Robinson, 1975). While, Gaal and Neujahr (1979), Katayama-Hirayama et al. (1991), and Paller et al. (1995) have described the metabolism of phenol by *Trichosporon cutaneum*, *Rhodotorula rubra* and *Acinetobacter calcoaceticus*, respectively, via an *ortho* cleavage pathway.

It should be pointed out that the information about the kinetics of phenol biodegradation is necessary for optimal performance. The inhibitory nature of phenol against the microbial growth at high and low concentrations is well known. Microbial growth can be inhibited in three ways: 1) by adding inhibitors, such as phenol, chlorine, benzenoid compounds, or antibiotics; 2) by increasing concentration of substrate to a level such that the growth decreases and ultimately ceases (substrate inhibition); 3) by allowing the metabolite concentration to a toxic level, such as alcohol fermentation (product inhibition) (Wayman and Tseng, 1976).

Sokoł (1987) found that in the microorganisms growing on an inhibitory substrate like phenol in a high conversion range, the total active enzyme is split between free enzyme and enzyme-substrate complex SE which breaks down to the product and fresh enzyme E. In addition, the level of enzyme present in microorganisms depends on the environmental substrate concentration, and is higher at higher values of the dilution rate. When the dilution rate approaches the wash-out dilution rate, the amount of free enzyme decreases.

At steady states, the enzyme concentration corresponds to the substrate concentration. In a batch culture, the substrate concentration is higher compared with the enzyme concentration, leading to inhibited enzyme-substrate complexes SES, which do not break down to product and free enzyme (substrate inhibition).

A variety of kinetic substrate inhibition models have been used to describe dynamics of the microbial growth on phenol. Of the various substrate inhibition models, the Andrews equation has been used extensively to describe phenol biodegradation (Andrews, 1968, Chi and Howell, 1976, Edwards et al. 1972, Hill and Robinson, 1975, Hutchinson and Robinson, 1988, Kotturi et al. 1991, Rozich and Colvin, 1986, Sokoł, 1988a, Sokoł and Howell, 1981 and Yang and Humphrey, 1975). The Andrews equation (Haldane) is based on the specific growth rate (Allsop et al. 1993 and D´Adamo et al. 1984), but may also be related to the specific substrate consumption rate (Edwards, 1970 and Sokoł, 1988b). Other kinetic models have been propagated. Sokoł (1988b), has shown a better fit for a modified Haldane equation but the kinetic parameters vary according to the "history"of the microorganisms. Allsop et al. (1993), reported a clear indication for an intermediate production during phenol degradation in continuous culture after applying a step increase in the substrate feed concentration. The authors suggest that the specific growth rate may not be a function of the substrate concentration at all during dynamic states and that intermediates must be considered even if substrate leakage is not detected. Wang and Loh (1999), found that the Haldane equation was not sufficient for modelling phenol degradation, especially for experiments at high initial phenol concentration, although it could correlate specific growth rate with initial substrate concentrations very well.

On the other hand, Schröder et al. (1997) who compared the fitting of kinetic data for phenol degradation by *Burkholderia cepacia* G4 derived from stationary conditions (from controlling of unstable steady states) with various kinetic equations and found the best fitting for the "Yano and Koga" equation. Phenol was transiently converted to an intermediate of 4-hydroxy-2-oxovalerate by *Burkholderia cepacia* G4 in continuous cultivation under pulse technique (Sipkema et al. 1998).

The toxicity of phenol for the cells was also investigated. Membranes of *Escherichia coli* cells grown in the presence of phenol were influenced. Keweloh et al. (1989 & 1990) found that both membrane types (inner and outer) showed reduced lipid-to-protein ratios compared with cells grown without phenol. An increase in membrane permeability is also induced by phenol and this occurs already at relatively low concentrations. Increasing of the respiratory activity of the cytoplasmic membrane was also observed under these conditions.

1. 1. 1. 2. Biological degradation of benzoate

Like phenol, benzoate is one of the aromatic compounds containing an aromatic moiety which ultimately undergoes enzymatic ring-cleavage. Benzoate and its derivates are widely used as fuels, food preservatives and industrial solvents. In addition, they provide the starting materials for the production of pharmaceuticals, agrochemicals, polymers, explosive and many other every day products (Gibson, 1971 and Smith, 1990). They are released into the environment through routine disposal in waste treatment facilities.

Benzoate is one of the simplest aromatic compounds and is a product of oxidative catabolism of many aromatic hydrocarbons (Hamzah and Al-Baharna, 1994). Recently, benzoate has been reported to be an intermediate of anaerobic benzene biodegradation (Caldwell and Suflita, 2000).

The fate of benzoate and its derivatives in the environment has been investigated. to existence of microorganisms able to degrade benzoate aerobically has been known for many years. As sole source of carbon and energy, benzoate can be used by many species of aerobic bacteria belonging to several different genera. Of them, *Alcaligenes eutrophus* (Ampe and Lindley, 1995 & 1996, and Stanier and Ornston, 1973), *Azotobacter sp*. (Sala-Trepat and Evans, 1971), *Comamonas acidovorans* (Groenwegen et al. 1992), *Pseudomonas aeruginosa* (Hickey and Focht, 1990 and van der Woude et al. 1995), *Pseudomonas cepacia* (Ghadi and Sangodker, 1994, and Hamzah and Al-Baharna, 1994) and *Pseudomonas putida* (Feist and Hegeman, 1969b, Nakazawa and Yokota, 1973, and Ornston, 1966).

The biological degradation of benzoate as sole source of carbon was investigated aerobically by *Burkholderia cepacia* G4 with relatively high conversion rates (Nelson et al. 1987). Although the catabolism of aromatic compounds by bacteria has been extensively studied, degradation of benzoate or its derivatives by yeasts and fungi has received less attention. Some yeasts are capable of mineralizing benzoate such as: *Candida sp*. (Hofmann, 1986), *Rhodotorula rubra* (Wright and Ratledge, 1991) and *Tricosporon sp*. (Gaal and Neujahr, 1980).

Benzoate degradation by *Pseudomonas putida* is well investigated. The bacterial attack on benzoate involves conversion of the aromatic nucleus to catechol. Once catechol is formed, it is consumed through catechol-2,3-dioxygenase (*ortho* cleavage pathway) which occurs in most strains. The route involves the cleavage of catechol to ß-ketoadipate, which is cleaved after activation to yield acetyl coenzyme A and succinate (Stainer et al. 1950, and Yamaguchi and Fujisawa, 1980 & 1982).

Benzoate as primary substrate induces only the enzymes responsible for its own conversion to catechol (Feist and Hegeman, 1969b). The *meta* cleavage pathway can be also induced for mineralization of benzoate (Harayama et al. 1987). Once it is formed, catechol converts via catechol-2,3-dioxygenase to 2-dydroxymuconic semialdehyde, which is characterized by its yellow colour.

The appearance of a yellow colouration of the medium during growth on benzoate has been described (Johnson and stanier, 1971). Up to now, no further evidence for the induction of the *meta* cleavage pathway has yet been reported despite the use of the appearance of a yellow colour as a test for the presence of this pathway.

Ironically, the cells of *Pseudomonas cepacia* did not grow on catechol as a sole source of carbon. This observation was reported by Feist and Hegeman (1969b) for strain mt-2 that degraded benzoate through the *meta* pathway. The same observation was also given by Hamzah and Al-Baharna (1994).

Hamzah and Al-Baharna (1994) have shown that the biodegradation of benzoate in *Pseudomonas cepacia* occurs interestingly through both pathways (*ortho* and *meta*) induced simultaneously. This strain posses the genetic capacity for enzymes of both *ortho* and *meta* cleavage pathways of benzoate degradation, although the phenotypic expression for the *orth* pathway is higher. Such findings indicate the extraordinary versatility and the unusual degradative potential of *B. cepacia*, which makes it a highly suitable bacterium for studying of the evolution of catabolic pathways and of the mechanisms by which they are regulated. The simultaneous induction of catechol-1,2-dioxygenase is not detected in salicylate degradation. Although it is the main metabolic intermediate for both salicylate and benzoate, catechol did not induce either pathway when used as a sole source of carbon. The same uncommon co-induction of both catechol-ring pathways was also reported for *Pseudomonas putida (arvilla)* mt-2 (Nakazawa and Yokota, 1973).

Müller et al. (1996), and Timmis et al. (1988) reported that the chlorosubstituted compounds are usually metabolized by the *ortho* cleavage route. Misriuting into the *meta* cleavage pathway leads to formation of toxic intermediates (suicide metabolism). Correspondingly, alkylbenzoates, which are normaly mineralized via the *meta* cleavage route, lead to production of dead end products if they are channelled into the *ortho* cleavage pathway.

The growth behaviour of some strains degrading benzoate or its derivatives using various substrate concentrations was investigated. Ampe and Lindley (1996) described the kinetic parameters of *Alcaligenes eutrophus* 335 growing on low and high concentrations of benzoate. The authors detected an inhibitory effect against growth at higher concentrations up to 40 mM. Despite the inhibitory effect, they used Michaelis-Mentens (Monod) equation to describe degradation of benzoate.

Recently, growth and metabolic pathways of *Pseudomonas putida* ATCC 49451on various sodium benzoate concentrations under batch cultivations were studied by Loh and Chua (2002). The results not only indicate that the degrading strain possesses both *ortho* and *meta* cleavage pathways of catechol for degradation of benzoate, but also that their regulation is related indirectly to the sodium benzoate concentration.

Like for *Pseudomonas cepacia*, the authors found that the cells of *Pseudomonas putida* ATCC 49451did not grow on catechol as a sole carbon source. This findings support observations of Feist and Hegeman (1969b) and Hamzah and Al-Baharna (1994). Eventually, a low initial concentration of sodium benzoate, the cell growth rate was described by the Monod model, but product inhibition was observed at higher initial concentrations (Loh and Chua, 2002). The inhibitory effect of benzoate was also observed on the growth of *E. coli* (Salmond et al. 1984).

Although catechol is the main intermediate for benzoate degradation in most bacterial strains, fungi convert benzoate to protocatechuate by two successive monohydroxylations with 4-hydroxybenzoate as an intermediate. The existence of the 2-oxoadipate pathway for the degradation of benzoate has been described (Cain et al. 1968, Gaal and Neujahr, 1980, Hofmann, 1986, and Wright and Ratledge, 1991). The inhibitory effect of benzoate on the growth of different yeast species were studied by MüncnerovÁ and Augustin (1994). They suggested the "Henderson-Hasselbach" equation as kinetic inhibitory model to describe the inhibitory effect of benzoate on the fungal growth.

Generally, the inhibitory effect exerted by benzoate is different from that described for the growth of *Pseudomonas putida* with phenol (Hill and Robinson, 1975 and Yang and Humphrey, 1975).

1. 1. 1. 3. Biological degradation of acetate

Acetate and its derivatives are compounds containing only two carbons. These compounds are minor toxic aliphatic substrates which can represent wastes of urban or industerial origin. Acetic acid belongs to the group of food preservatives and is widely used as industrial solvents. Based on the breakdown of the organic matter, acetate is considered a key intermediate in anaerobic bioprocesses. Under the conditions for treating sulfate-rich wastewaters such as paper mill and food oil industry wastewaters, sulfate reducers compete for these compounds with methanogens (Oude Elferink et al. 1994).

Under aerobic conditions, different microorganisms are capable to use acetate as a sole source of carbon and energy. Pseudomonads grow more rapidly on acetate like *Pseudomonas* KB1 (Kornberg and Madsen, 1957), *Pseudomonas* M27 (Norbad et al. 1989) and *Pseudomonas fluorescens* 23F (Hernandez and Johnson, 1967, and McMullan and Quinn, 1994). Additionally, the acetate was degraded very well by *Escherichia coli* (Holms, 1987 and Hoyt et al. 1988).

Ampe and Lindley (1995) who investigated degradation of benzoate, used *Alcaligenes eutrophus* 335 also to degrade acetate under the same conditions. By *Comamonas testosteroni* acetate was utilized (Gerritse et al. 1992). Moreover, *Candida utilis* and *Saccharomyces carlsbergensis* were grown on acetate and on several carbon sources by Hernandez and Johnson (1967).

Regulation of acetate metabolism under aerobic conditions was described. Kornberg and Madsen (1957) reported that if microorganisms grow on two carbon compounds like acetate or ethanol, the "glyoxylate cycle", an auxilary pathway, is active in addition to the TCA. It provides an alternative route from isocitrate to malate to provide metabolic intermediates used directly for the synthesis of other cell constituents and also when incomplete oxidation occurs. Hoyt et al. (1988), mentioned that the glyoxylate cycle was first discovered during studies on bacteria and fungi with the ability to grow on acetate or ethanol as the sole carbon source and the isocitrate lyase was the first enzyme unique to the glyoxylate cycle in numerous pro-and eukaryotic organisms.

Cozzone (1998) and Noronha et al. (2000) have described and simplified the operation of both TCA cycle and the glyoxylate shunt at three cyclic pathways starting with oxaloacetate as follows:
1. **TCA:** Oxaloacetate ➛ Citrate ➛Isocitrate ➛Ketoglutarate ➛Succinate ➛Malate ➛Oxaloacetate.
2. **Glyoxylate-Succinate:** Oxaloacetate ➛Citrate ➛Isocitrate ➛Succinate ➛Malate ➛Oxaloacetate.
3. **Glyoxylate-Malate:** Oxaloacetate ➛Citrate ➛Isocitrate ➛Glyoxylate ➛Malate ➛Oxaloacetate.
Accordingly, the presence of a glyoxylate bypass has been reported in all organisms that can employ acetate or fatty acids as their sole carbon source. Evolution of the glyoxylate bypass was obtained by Holms (1987), using *Escherichia coli* when acetate is the sole source of carbon and energy under aerobic conditions.

Analysing the kinetic parameters of acetate biodegradation indicated that the simple "Michaelis-Menten" equation was sufficient to describe the growth behaviour of many microorganisms on acetate aerobically (Gerritse et al. 1992).

The dynamic behaviour of *Saccharomyces cerevisiae* growing on acetate was successfully modelled under aerobic conditions (Duboc et al. 1998). The mechanistic model considers the decoupling between the anabolic and catabolic activities. After a sudden increase in the dilution rate, the dynamic response depends upon the ability of the culture to adapt to the new growth conditions. The authors observed that the adaptation of the metabolism to a higher substrate supply is not immediate. Usually, the catabolic activity increases more rapidly as the biomass formation rate.

1. 1. 2. Cultivation of the substrate mixtures

Mixtures of organic chemicals are prevalent in wastewaters from industerial and municipal sources as well as in contaminated groundwater. Common examples of chemical mixtures that often become pollutants include gasoline and other petrolium fuels, pesticides and wood-treating substances. The organic mixtures may contain only organic chemicals or may also include inorganics, heavy metals, or radionuclides.

Occurrence of the environmental contaminants in mixtures is an important problem because the removal or degradation of one component can be inhibited by other compounds in the mixture, and because different conditions may be required to treat different compounds within the mixture (Readon et al. 2000).

Industrial production of toxic organic chemicals and its widespread use in various applications has led to extensive environmental contaminations in the recent decades. It was also noticed that the toxic compounds often entered the environment by industrial effluents and spills. Analysis of the contaminated soils has shown that the toxic compounds can sometimes persist for a long time even in the presence of microorganisms that are capable of mineralizing them. Regarding the growth conditions, environment of the microorganisms is not often suitable for rapid degradation (Hecht et al. 2000).

Currently, quantitative information on the rates and patterns of microbial utilization of substrate mixtures is scarce. The microbial utilization of substrate mixtures is important not only in biotechnology applications, such as the production of fuels from biomass and the use of microbs to produce secondary metabolites in pharmaceutical applications (Lendenmann, 1994), but also in the biodegradation of hazardous chemicals (Rogers and Readon, 2000).

In general, the microbial degradation (metabolism) of a compound in a mixture can be strongly influenced by other compounds of the mixture (Egli, 1995 and Saéz and Rittmann, 1993). This has been observed not only for mixtures of toxic chemicals (bioremediation), but also for mixtures of pollutants and readily degradable compounds (wastewater treatment), and mixtures of sugars (fermentation). Moreover, biodegradation of individual pollutants in mixtures has been shown to be different from the degradation as single carbon source (Readon et al. 2000 and Smith et al. 1991).

To overcome unsuitable or non ideal conditions in the environment, bioreactors have been used successfully to mineralize various toxic or inhibitory compound mixtures, such as BTEX (Bielefeldt and Stensel, 1999, and Shim and Yang, 1999), quinoline (Buchtmann et al. 1997), dichloroaniline (Livingston, 1991), substituted benzoic acids (Müller et al. 1996), and phenolic componds (Anselmo and Novais, 1992). In bioreactors, control of the microenvironment of the cells is easier than in the soil, and high degradation rates can be achieved (Léonard et al. 1999).

If a microbial population is grown on a mixture of substrates, any of which can be used as a carbon and energy source, several utilization patterns can be observed. The utilization pattern is depending mainly on substrate concentrations, oxygen concentration and microbial growth rates, and can change with different mixture compositions. For the sequential utilization pattern, characterized by the "diauxi" phenomenon, a compound can inhibit the degradation of another by exerting toxicity, catabolite repression, competitive inhibition for enzymes, or depletion of electron acceptors (Smith et al. 1991).

The other pattern of substrate mineralization in a mixture is the simultaneous utilization, in which, the removal efficiencies are enhanced compared with growth on the individual compounds. This enhancement has been attributed to the production of higher growth rates (Klečka and Maier, 1988). This mechanism is due to either the nonspecificity of enzyme induction for degradation of similar substrates or the convergence of catabolic pathways for the utilization of many substrates. Even though, the compounds are degraded simultaneously, but usually they are not degraded at the same rate.

Biodegradation of substances classified as toxic or hazardous pollutants in the presence of non-toxic, easily biodegradable, organic compounds is a topic which has attracted the attention of many investigators. The interest in this topic is mainly for wastewater treating facilities involving wastes from different origins. For example, wastes originating from industerial sources may containing hazardous or toxic substrates, whereas wastes from urban or agricultural sources usually containing non toxic pollutants.

In the presence of alternative substrates, degradation of phenol was investigated in different mixtures. Using a pure culture of *Pseudomonas acidovorans*, phenol and acetate were utilized simultaneously in their binary mixture (Schmidt and Alexander, 1985), with diminishing of phenol degradation. This was the first demonstration that aromatic pollutants can be utilized simultaneously with other compounds by pure cultures of bacteria. The metabolism of aromatic compounds by members of the genus *Pseudomonas* is usually repressed by the presence of more easily degradable compounds, especially intermediates of the tricarboxylic acid cycle (Weitzman and Jones, 1968). Repression of catechol-2,3-dioxygenase required to degrade catechol was observed in the presence of acetate, benzoate and p-toluene using *Alcaligenes eutrophus* 335 (ATCC 17697) (Hughes and Bayly, 1983).

On the other hand, effect of glucose against degradation of phenol was studied. Using a heterogeneous population and mixtures of phenol and glucose, Rozich and Colvin (1986) observed simultaneous utilization of both compounds. Recently, Wang et al. (1996) found that a simultaneous utilization of both substrates by *Pseudomonas putida* (ATCC 17514) was also observed, but with lower specific rates. Reduction of the specific substrate utilization rates indicates that the two substrates are involved in a cross-inhibitory pattern which can be classified as uncompetitive. The inhibitory effect between phenol and glucose was also obtained by *Burkholderia cepacia* G4 (Kehlenbeck, 1998).

A simultaneous utilization pattern was also obtained for phenol and p-cresol under batch conditions by *using Pseudomonas putida* (ATCC 17484). The data could be described by a simple model implying no interaction between the two pollutants (Hutchinson and Robinson, 1988). Both substrates were metabolized via a catechol meta cleavage pathway by *Alcaligenes eutrophus* 335 (ATCC 17697) (Hughes and Bayly, 1983 and Johnson and Stanier, 1971).

The biodegradation of phenol in the presence of the aromatic compound toluene was also investigated. Degradation of both substrates in their mixture using *Burkholderia species* JS150 followed simultaneous utilization kinetics with toluene being the first substrate completely degraded. Toluene was found to inhibit removal of phenol, while presence of phenol had little effect on the rate of toluene degradation (Rogers and Readon, 2000). These observations provide an interesting contrast to observations of sequential utilization of phenol-toluene mixtures by *Pseudomonas putida* F1 (Readon et al. 2000). This type of simultaneous utilization is evidence that the same metabolic pathway was used in the degradation of both substrates.

Since *Burkholderia species* JS150 contains both *ortho* and *meta* pathways for the degradation of phenol (Haigler et al. 1992), and only the *meta* pathway is utilized for the degradation of toluene, these results indicate that the *meta* cleavage pathway might be preferentially used over the *ortho* cleavage pathway for this situation.

In a fluidized-bed reactor, binary mixtures of phenol and benzoate were also successfully degraded by *Burkholderia cepacia* G4 (Hecht et al. 2000). In addition, the culture suspension was colourless at all conditions used. The yellow ring cleavage product of the *meta* cleavage pathway, 2-HMS, was therfore not accumulated in the culture suspension. On the other hand, degradation of a phenol-benzene mixture by *Pseudomonas putida* F1showed a preference of benzene, whereas phenol consumption did not begin until benzene concentrations were near zero (Readon et al. 2000). It seems evident from these observations, that different microorganisms can lead to totally different utilization patterns for a given substrate mixture.

Since soil and water polluted with a toxic organic compound can sometimes contain lots of other toxic compounds, the biodegradation of certain contaminants can be significantly influenced by the presence of other contaminants. To degrade two toxic compounds, immobilized *Nocardiodes sp*. NSP41was grown on a binary mixture of phenol and p-nitrophenol as carbon and energy source (Cho et al. 2000). The authors stated that the degradation of both toxic substrates in industrial wastewaters is feasible because both compounds were degraded simultaneously with high consumption rates.

Under more complex conditions, degradation of a ternary substrate mixtures comprising 4-chlorophenol and sodium glutamate in addition to phenol as carbon sources using *Pseudomonas* putida ATCC 49451was investigated by Wang and Loh (2000 & 2001). The effects of substrate interactions, in addition to kinetic modelling were studied. The authors found cross-inhibitions among all three substrates which were degraded simultaneously.

Growth of *Pseudomonas putida* F1 on a three-substrate mixture of phenol, benzene and toluene was also studied by Readon et al. (2000). As a result, all substrates were metabolized by the same enzymes and toluene was the preferred substrate.

In contrast to the laboratory, growth in ecosystems proceeds under more complex conditions where microorganisms are faced with mixtures of compounds that can fulfill a particular nutritional function. This is probably best illustrated for heterotrophic microbial cells and the carbon substrates they use for growth (Marita, 1988). The catabolic versatility and flexibility of microbial cells for growth under limited conditions of carbon and energy and for growth in carbon sufficient batch cultures was demonstrated by Egli, 1995. This raises the question whether the traditional kinetic concepts based on constant kinetic and stoichiometric parameters can be applied to different environmental situations originating out of a different culture history (Kovárová-Kovar and Egli, 1998).

In substrate mixtures, specific rates are altered compared with degradation of single substrate systems. Reduction of specific substrate consumption rate was observed for different substrate mixtures, which was probably caused by changes in the actual conversion rates of the studied substrates and/or changes in their degradation ability (Singirtsev et al. 2000). On the other hand, an increase in the specific growth rate was observed when a culture was exposed to mixtures containing three or more carbon sources compared with growth on either of these substrates as single carbon sources (Brinkmann and Babel, 1992, Lendenmann and Egli, 1998, and Reber and Kaiser, 1981).

It is worth noting, that catechol occurs as an intermediate in the metabolism of both phenol and benzoate by *Burkholderia* (*Pseudomonas*) *cepacia* G4. Cleavage of the aromatic ring of catechol may, in general, proceed either by the *ortho*-or by the *meta*-pathway. For strain G4, the use of the *meta*-pathway is described (Nelson et al. 1987). Fig. (1. 1) shows the *meta* cleavage pathway and the routes to degrade phenol, benzoate, and acetate, to acetyl CoA.

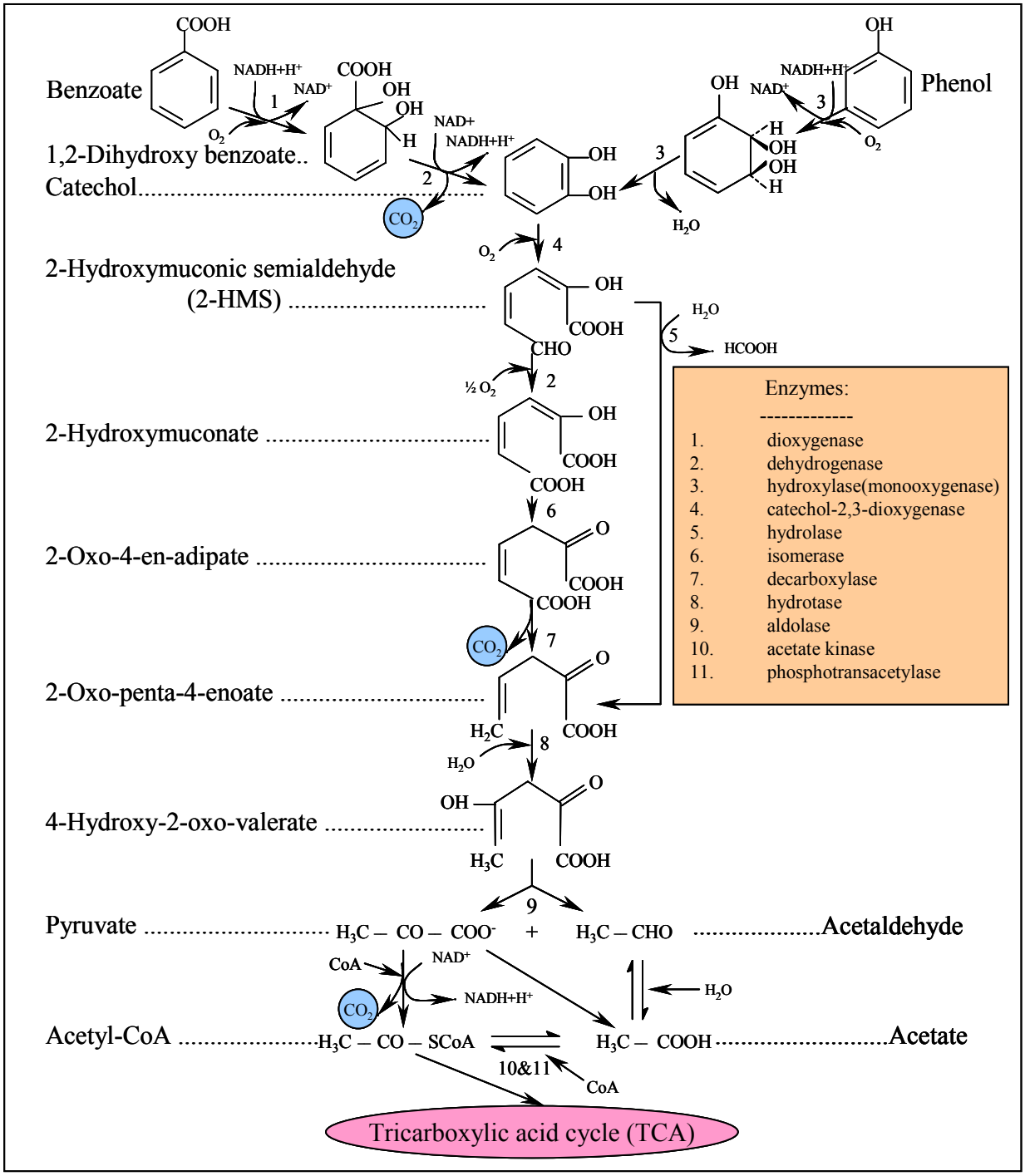

Fig. 1. 1. Metabolites and enzymes of *meta*-cleavage pathway for the biodegradation of phenol (Feist and Hegeman, 1969a, and Nelson et al. 1987), benzoate (Hamzah and Al-Baharna, 1994), and acetate to acetyl-CoA (Cozzone, 1998).

1. 2. Aim of the work

The general aim of the presented work is to investigate the performance of the microbial degradation of toxic organic pollutants (xenobiotics) in the presence of non-toxic organic compounds which commonly exist in the environment and which may hamper or lower the conversion of the toxic compounds. The model system used is the degradation of phenol, benzoate and acetate by a pure culture of *Burkholderia (Pseudomonas) cepacia* G4. Special emphasis will be directed towards the degradation kinetics, which will be compared for single and mixed substrate conversion and to the constancy or variability of the kinetic parameters. It is hoped that the presented work will contribute to a better understanding of the degradation kinetics in general and of the complex system behaviour in mixed substrate systems.

In order to realise the aim mentioned above, this work is focussed on the following main tasks:

I. Investigating the biological degradation of the selected substrates under carbon-limiting conditions.

- Estimating the stoichiometric parameters from cultivation of the substrates studied individually and in mixtures using different dilution rates under chemostat conditions.
- Confirming whether the stoichiometric behaviour of the mixture can be expressed by the data obtained from cultivation of the single substrate systems.
- Evaluating the dependence of the steady state on the concentration of the particular substrate (mixture composition) in the medium feed compared with the cultivations of single substrates.

II. Studying the biological degradation of the three substrates under different dynamic conditions.

➢ Analysing the single substrate systems:

- Evaluating the growth dynamics by using different techniques (batch, wash-out, and A-stat), and by using different initial substrate concentrations (batch).
- Comparing the stoichiometry and kinetics derived from different cultivation methods.

➢ Analysing the mixed substrate systems:

- Investigating the growth behaviour on different types of substrate mixtures using different initial substrate concentrations for batch experiments, and comparing the results with the single substrate experiments.

- Investigating interaction between substrates in substrate mixtures, as well as studying their utilization pattern using different initial substrate concentrations.

- Comparing single and multi-substrate experiments in regard with their performance, growth dynamics and stoichiometry.

2. MATERIALS AND METHODS

2.1. Microorganism

Burkholderia (Pseudomonas) cepacia G4 was used in this study. The strain is characterized as a non-motile, gram-negative and rod-shaped bacterium which grows predominately in pairs and short chains in logarithmic phase (Nelson et al. 1986). *Burkholderia cepacia* G4 was a gift of K. N. Timmis (Division of Microbiology, GBF, Braunschweig, Germany). The elemental composition for biomass of the studied strain was assumed to be $CH_{1.79}O_{0.50}N_{0.20}$ (Roels, 1980). The ash content was considered as 7.5 % (average of various organisms).

2. 2. Culture media

The microorganism was cultivated in a mineral salt medium containing per liter: 894.0 mL deionized water, 100.0 mL buffer solution, 1.32 mL trace elements solution, 0.68 mL solution A and 4.0 mL solution B. Compositions of each solution were as follows:

*** Buffer solution composition:**

Na_2 HPO4 . $2H_2O$ ..... (Riedel de Haen 30412).....87.78 g L^{-1}
KH_2PO_4 .....(Riedel de Haen 04243).....30.00 g L^{-1}
$(NH_4)_2$ SO_4 ..... .(Riedel de Haen 30620).....12.37 g L^{-1}

*** Trace elements solution composition:**

Mg O ..... (Merck 5867).....10.75 g L^{-1}
Fe SO_4 .$7H_2O$ ..... (Merck 3965) .....04.50 g L^{-1}
Ca CO_3 .....(Merck 2066).....02.00 g L^{-1}
Zn SO_4 . $7H_2O$ .....(Merck 8882) .....01.44 g L^{-1}
Mn SO_4 . $2H_2O$ ..... (Merck 5959) .....00.87 g L^{-1}
Co SO_4 . $7H_2O$ ..... (Merck 2556) .....00.28 g L^{-1}
Cu SO_4 . $5H_2O$ ..... (Merck 2790)..... 00.25 g L^{-1}
H_3 BO_3 ..... (Merck 165) .....00.06 g L^{-1}
HCl (37 %) .....(Riedel de Haen 30721) .....51.30 mL

*** Solution A:**

Mg SO_4 .....(J.T.Baker 0168).....246.48 g/L = 1 mol L^{-1}

*** Solution B:**

$Fe\ SO_4 . 7H_2O$ ....(Merck 3965).....03.20 g L^{-1}
EDTA [Ethylene Diamine Tetraacetic Acid]..... (Merck 8418).....09.45 g L^{-1}

The medium was prepared by autoclaving water and buffer solution. The pH was adjusted at 7.0. The trace elements solution and solutions A and B were filter sterilized (AQ 0749-1, red rim, Renner GmbH, Dannstadt, Germany) and added aseptically to the autoclaved part after cooling to prevent precipitation.

2. 3. Cultivation System

2. 3. 1. Cultivation in shake flask

Flask experiments were done in 500 mL Erlenmeyer flasks containing autoclaved 223.5 mL deionized water and 25 mL buffer solution. A mixture of 0.33 mL trace elements solution with 0.17 mL solution A and 1.0 mL solution B was filter sterilized (AQ 0749-1, red rim, Renner GmbH, Dannstadt, Germany) and added aseptically under sterile conditions to the autoclaved part after cooling to prevent precipitation.

The microorganism was cultivated in 500 mL Erlenmayer flasks with 250 mL liquid culture. The cultures were incubated at 30˚C with rotary shaking at 120 rpm and maintained on phenol solution (4 mL phenol 0.5 mol per week) as a sole carbon and energy sources .

2. 3. 2. Cultivation in bioreactor

For the continuous cultivations, 50 L sterile medium was prepared in 60 L tanks made from stainless steel. The composition of this medium was 44.7 L deionized water, 5 L buffer solution, 33.5 mL trace elements solution, 66.5 mL solution A and 200 mL solution B. The mixture was sterile filtered (AQ 0749-1, red rim, Renner GmbH, Dannstadt, Germany) into a second previously autoclaved 60 L tank.

In addition, 50 L of stock solution from each studied substrate (phenol, benzoate, or sodium acetate) was prepared in 60 L stainless steel tanks. For preparation of a 5 g L^{-1} phenol feed solution, 250 g of the highest purified phenol was dissolved in deionized water to obtain 5 L concentrated solution. Accordingly, 500 g of the highest purified benzoate and sodium acetate were dissolved in the same volume of the deionized water for preparation of 10 g L^{-1} feed solutions of both substrates. The concentrated solutions were mixed with 39.7 L deionized water, 5 L buffer solution, 33.5 mL trace elements solution, 66.5 mL solution A and 200 mL solution B. After mixing, the prepared feed solutions were sterile filtered (Satoclean GF, Sartorius GmbH, Göttingen, Germany) in previously autoclaved 60 L steel tanks.

2. 4. Construction and periphery of the reactor

Fig. (2. 1) shows a schematic diagram of the experimental bioreactor set-up and the control units used throughout this study .

2. 4. 1. Reactor

All cultivations were carried out in a steam sterilizable 3.5 L stirred tank bioreactor (FZ 2000, Chemap AG, Volketswil, Switzerland). The working volume was 2.52 L. The reactor was equipped with a console for regulation of temperature, pH value, and agitation.

All cultivations were carried out at 25˚C by an external thermostat (RE 106, Lauda GmbH, Germany). The thermostat system includes an electric heater and a valve for cooling water supply.

The pH was measured by an autoclavable pH electrode (Ingold, Urdorf, Switzerland) and maintained at 7.0 by automatic addition of 1.0 mol L^{-1} sodium hydroxide solution and 0.5 mol L^{-1} sulfuric acid solution. The pH electrode was calibrated before sterilization and the pH values were checked routinely during cultivation by sampling.

Dissolved oxygen content (DO_2) was also measured by heat sterilizable electrode (Ingold, Urdorf, Switzerland). The electrode was calibrated after sterilization using nitrogen and air.

Aeration was done with compressed air at a flow at 162 L h^{-1} (STP) by using a mass flow controller (PR-3000, MKS, Germany). The inlet gas was sterilized with membrane filter (Cartridge filter, Pall Europe Limited, Portsmouth, England) and the stirrer speed was adjusted at 300 rpm .

The exhaust gas from the reactor was analysed for oxygen and carbon dioxide content (Servomex 1100 and 1400, respectively, Bühler GmbH, Ratingen, Germany) using the paramagnetic properties of oxygen and infrared (IR) absorption for CO_2 measurement. The accuracy of the CO_2 analyser is given as $\leq \pm 1\%$ of full scale or $\leq \pm 0.02\%$ CO_2, respectively.

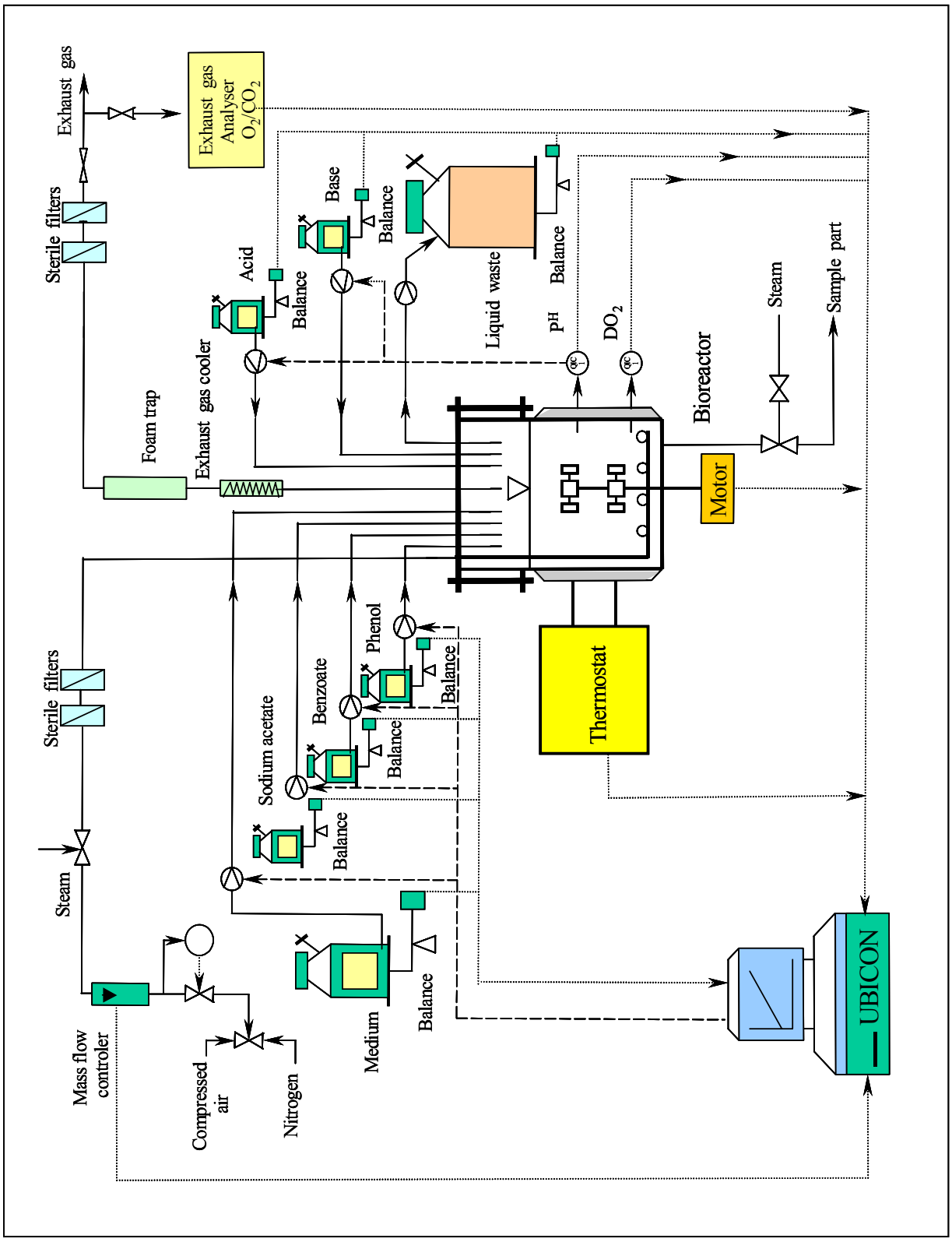

Fig. 2. 1. Schematic diagram of the bioreactor set-up.

2. 4. 2. Bioprocess control system

All pumps, balances, and the reactor main console were connected with a bioprocess control computer type UBICON (Universal Bioprocess Control System) (ESD, Hannover, Germany) for data acquisition and control strategies of the reactor.

2. 4. 3. Cultivations

For all continuous cultivations , the sterile medium and the concentrated substrate solutions were fed by 503 U peristaltic pumps (Watson Marlow, Falmouth, United Kingdom) into the bioreactor. A 503 U peristaltic pump was also used to with-draw culture broth by an overflow device to maintain a constant culture volume. The flow rates of the feed pumps were set automatically via UBICON according to a present dilution rate. Steady state data were assessed after waiting for at least five residence times ($\tau = D^{-1}$) at the operating conditions.

All batch cultivations were carried out of a chemostat. Prior to the batch experiment, the feed and harvest pumps were stopped and a concentrated substrate solution was added to the culture suspension through a sterile filter (Rezist 30 / 0.2 μ m, white rim, Schleicher and Schüll, Dassel, Germany) by a syringe via a membrane seals part. This procedure guarantee a defined and reproducible culture history for each experiment (adjusted batch). After finishing the batch experiment, the feed and harvest pumps were restarted.

For wash-out cultivations, dilution rate of the culture was stepped-up to a value higher than the maximum specific growth rate. For ramp cultivations (A-stat), the dilution rate was continuously increased via the computer-controlled program (UBICON).

2. 5. Analytical methods

The experimental methods used in the present work include determination of biomass, substrate, 2-hydroxymuconic semialdehyde concentrations and the exhaust gas. For off-line analytical methods, 25 mL samples were taken from the bioreactor. After harvesting of each sample, the sample port, located at the bottom of the rector, was steam sterilized.

2. 5. 1. Determination of biomass

For determination of biomass concentration as dry weight, two 10 ml aliquots were centrifuged for 15 min at 15000 rpm (24652 g) at a temperature of 15˚C (Biofuge Stratos, Heraeus instruments, Kendro, Hanau, Germany) in stainless steel tubes.

The pellets were dried at 60˚C for 48 h under vacuum (VT 6025, Heraeus vacutherm, Kendro, Hanau, Germany), cooled in an exsiccator at room temperature and weighted. The tubes were washed, dried and re-weighted empty. The difference between the first and second weight was used to calculate to determine the dry weight of biomass as g L^{-1} .

2. 5. 2. HPLC-analytical parameters

To determinate concentrations of the substrates in the reactor, about 5 mL of the sample were filtered using a sterile filter (Rezist 30 / 0.2 μ m, white rim, Schleicher and Schüll, Dassel, Germany) to remove the biomass. The filtrate was cooled and stored in small glasses at -20˚C for subsequent analysis. Concentrations of the substrates were quantified by using HPLC (High Performance Liquid Chromatography). According to the nature of the determinated substrate, different parameters were used:

∗ For determination of phenol

Detector : Biotronik UV-Detector BT 3030.
Detection : UV-Absorption λ =270 nm.
HPLC-pump : Abimed / Gilson HPLC-pump model 307.
Flow : 1.0 mL min^{-1}.
Reversed-phase column : Nucleosil 120-3C_{18} (721721.46) ; front column(721606.40).
Mobile-phase (solvent) : Methanol : water (6 : 4).
Temperature : Room temperature.
Elution : Isocratic.
Data analysis : Gilson Control Software 715.

∗ For determination of benzoate and acetate

Detector : Jasco , UV-970 (intelligent UV/VIS Detector).
Detection : UV-Absorption λ =210 nm.
HPLC-pump : Jasco , HPLC-pump , model PU-980.
Flow : 1.0 mL min^{-1}.
Reversed-phase column : Chromasil 100-5C_8 (728043.40) ; front column(728057.40)Chromcart.
Mobile-phase (solvent) : a) 100 mmol trikaliumphosphate monohydrate $K_3 PO_4 . H_2O$.
b)Water (Milli-Q) .
c) Methanol .

Composition of the mobile phase (solvent) used to determinate benzoate and acetate as function of the time are given in Table (2. 1).

Table 2. 1. Composition of the mobile-phase (solvent) used to determinate benzoate and sodium acetate during HPLC-analysis.

Time	Flow	Mobile-phase (solvent) %		
h	mL/min	a	b	c
0	1.0	100	0	0
2.4	1.0	100	0	0
5.0	1.0	20	80	0
8.0	1.0	10	40	50
12.0	1.0	10	40	50
15.0	1.0	20	80	0
18.0	1.0	100	0	0
25.0	1.0	100	0	0

Elution : Gradient.
Oven-temperature : 23˚C.
Data analysis : Jasco, Borwin Chromatography Software.

Benzoate concentrations were expressed as benzoic acid, while acetate concentrations were expressed as sodium acetate.

2. 5. 3. Analysis of 2-hydroxymuconic semialdehyde (2-HMS)

Formation of 2-hydroxymuconic semialdehyde (2HMS) was followed by measuring absorption using a spectrophotometer (Ultrospec 3300 pro, Amersham Pharmacia Biotech GmbH, Freiburg, Germany) at 385 nm. The samples were filtered using a sterile filter (Rezist 30 / 0.2 μ m, white rim, Schleicher and Schüll, Dassel, Germany) to remove the bacterial cells and the absorption of the supernatant was measured. If, the absorption was higher than 0.15, the samples were diluted as 1 : 10 and measured again. The values were divided by 16 for calculation of the molar concentration of 2-hydroxymuconic semialdehyde in the medium (D. Pieper, Division of Biodegradation, GBF, Braunschweig, Germany).

2. 5. 4. Effluent gas

The gas analyzer was calibrated using a reference gas mixture of known composition (19.1 % O_2 and 1.79 % CO_2) before and approximately once a week during longer cultivations. Gas content data were gathered on-line by the process control computer UBICON.

2. 6. Computational methods

For numerical solving of differential equations, simulations, and data fitting, the MATLAB (The Math Works Inc., Natick, MA, USA) software package was used in combination with the BIOSS-toolbox (written by K. Gollmer, Umwelt-Campus, Fachhochschule Trier). The BIOSS-toolbox is a MATLAB-based program package for modelling, simulation and optimization of dynamic processes. Incorporated is an algorithm for calculating sensitivity differential equations for parameter sensitivity investigations.

3. THEORETICAL BASIS

3. 1. Mass balances

The mass balance for a compound i in an ideal mixed reactor, which was considered for the stirred tank bioreactor used in this work, can be expressed as:

$$d\,(VL\,C_i) / dt = F_L^{\,in}\,C_{i\,0} - F_L^{\,out}\,C_i + V_{L\,(t)}\,(q^{mt} + q^r) \qquad [3.\,1]$$

with:
- V_L rector liquid volume at time t (L)
- C_i concentration of compound i ($g\,L^{-1}$)
- $C_{i\,0}$ concentration of compound i in feed ($g\,L^{-1}$)
- F_L convective liquid flow ($g\,L^{-1}$)
- q^{mt} gas/liquid mass transfer rate ($g\,L^{-1}\,h^{-1}$)
- q^r vector of biological reaction ($g\,L^{-1}\,h^{-1}$).

q^{mt} is usually referred to the oxygen or carbon dioxide transfer rate, OTR or CTR, respectively. The reaction rate vector q^r is positive for sources and negative for drains and is expressed as:

$$q^r = \sum r_i\,C_X \qquad [3.\,2]$$

Batch cultivation:

a batch cultivation is characterized by $V_{L\,(t)}$ = constant and $F_L = 0$. The mass balance for dissolved non gaseous compounds is defined as:

$$d\,C_i / dt = q^r \qquad [3.\,3]$$

and for dissolved gaseous compounds as:

$$d\,C_i / dt = q^{mt} + q^r \qquad [3.\,4]$$

Continuous cultivation:

A contineous cultivation is characterized by a steady feed of dissolved substrate into the reactor and an equal drain of culture suspension so that the reactor volume remains constant ($V_{L\,(t)}$ = const.). With the definition of the dilution rate D:

$$D = F_L / V_L\,, \qquad [3.\,5]$$

the mass balances for dissolved non volatile and gaseous compounds can be written as:

$$d C_i / dt = D (C_{i\,0} - C_i) + q^r \quad [3.\ 6]$$

$$d C_i / dt = D (C_{i\,0} - C_i) + q^r + q^{mt} \quad [3.\ 7]$$

respectively. For dissolved oxygen and carbon dioxide at dilution rates not too high, the convective flow is usually neglected. At steady state, the differential equations are zero and the mass balances for substrate and biomass, for example, are:

$$\mu = D \quad [3.\ 8]$$

and

$$r_S C_X = D (C_{S\,0} - C_S) \quad [3.\ 9]$$

with:

C_X	biomass concentration (g L^{-1})
C_S	limiting substrate concentration (g L^{-1})
$C_{S\,0}$	substrate concentration in the feed (g L^{-1})
D	dilution rate (h^{-1})
μ	specific growth rate (h^{-1})
r_S	specific substrate consumption rate (g g^{-1} h^{-1})

3. 2. Kinetics

The kinetic equation for a compound i describes the influence of the concentration C_i and of other compounds (inhibitors or activators) on the conversion rate r_i. The most important work about microbial growth kinetics was done in 1942 by Monod. For substrate limited growth he proposed this following equation:

$$\mu = \mu_{max} C_S / (K_S + C_S) \quad [3.\ 10]$$

with:

μ_{max}	maximum specific growth rate (h^{-1})
K_S	substrate affinity constant (g L^{-1})

At low concentrations, the specific growth rate resembles a fist order kinetics ($K_S >> C_S$), at high concentrations a zero order kinetics ($K_S << C_S$). The equation was derived from the well known Michaelis-Menten equation of enzyme kinetics and is often used in the Michaelis-Menten way to express substrate conversion kinetics:

$$r_S = r_{S\,max}\, C_S / (K_S + C_S) \qquad [3.\ 11]$$

with: $r_{S\,max}$ maximum specific substrate consumption rate (g g^{-1} h^{-1}).

There are several other kinetic expressions incorporating influence of other compounds like biomass, ..etc., most of them are derivations of the Monod equation. A review is given by Bitton and Koopman (1986) and Marison (1988). The Monod or related equations describe substrate limited growth or conversion. In case of the presence of an inhibitor, one must distinguish between product and substrate inhibition. Product inhibition is usually described by the following equations which were also derived from enzyme kinetics (Segel, 1975):

- competitive inhibition, in which the inhibitor and the substrate compete for the same reactive site on the enzyme:

$$r_S = r_{S\,max}\, C_S / (K_S\,(1 + C_i / K_i) + C_S) \qquad [3.\ 12]$$

- non competitive inhibition, in which the inhibitor can bind to both the enzyme and the enzyme-substrate complex:

$$r_S = r_{S\,max}\, C_S / ((K_S + C_S)\,(1 + C_i / K_i)) \qquad [3.\ 13]$$

- uncompetitive inhibition, in which the inhibitor binds to the enzyme-substrate complex, but not to the free enzyme:

$$r_S = r_{S\,max}\, C_S / (K_S + C_S\,(1 + C_i / K_i)) \qquad [3.\ 14]$$

with: C_i concentration of inhibitor (g L^{-1})
K_i inhibition constant (g L^{-1})

There are, of course, several other equations published in the literature (Allsop et al. 1993, Kovárová-Kovar and Egli, 1998, Luong, 1987, Schröder et al. 1997, Sokoł, 1988a, ..etc.). Substrate inhibition is most often expressed by the Andrews equation (equal to the Haldane equation of enzyme kinetics):

$$\mu = \mu_{max}\, C_S / (K_S + C_S + C_S^{\,2} / K_i) \qquad [3.\ 15]$$

or

$$r_S = r_{S\,max}\, C_S / (K_S + C_S + C_S^{\,2} / K_i) \qquad [3.\ 16]$$

There are also several other published equations describing substrate inhibition kinetics, some of them derived from the Monod equation (Wayman and Tseng, 1976), some not (Tan et al. 1996). The Andrews equation can also be obtained from uncompetitive product inhibition if the inhibitory product concentration is replaced by the substrate concentration. A very versatile equation for substrate inhibition was proposed by Yano and Koga (1969):

$$r_S = r_{S\,max} C_S / (K_S + C_S + C_S \sum_{j=1}^{p} (C_S / K_i)^j \qquad [3.\ 17]$$

Here, the formation of inactive enzyme-substrate-complexes with more than two substrate molecules is considered. The Andrews or Haldane equation is obtained by j =1. The Yano and Koga equation was used by Schröder et al. (1997) to describe phenol conversion by *B. cepacia* G4 in continuous culture, giving a better data fit than several other tested equations.

In general, the kinetic coefficients are considered as constant. However, in the newer literature this is doubted (Kovárová-Kovar and Egli,1998).

3. 3. Stoichiometry

In general, the relationship between growth, substrate consumption, and product formation is expressed by yield coefficients. Yield coefficients calculated from experimental data are not constant but subject to the cell maintenance. The basis work was done by Pirt (1965). The yield coefficients are usually calculated from a plot of the specific consumption or production rate as a function of the specific growth rate. In a continous cultivation at steady state the specific growth rate equals the dilution rate D. From the slope of a plot $r_j = f(D)$, the 'true growth' yield is obtained: slope $=Y_{X/j}^{-1}$:

$$r_j = (D/Y_{x/j}) + m_j \qquad [3.\ 18]$$

The relationship between the experimental and the 'true growth' yield is given by:

$$1/Y_{x/j}^{EXP} = (1/Y_{x/j}) + m_j / D \qquad [3.\ 19]$$

with:
- $Y_{X/j}$ 'true growth' yield coefficient of compound j (g g^{-1})
- $Y_{X/j}^{EXP}$ 'experimental' yield coefficient of compound j (g g^{-1})
- m_j maintenance coefficient for compound j (g g^{-1} h^{-1})

Equation [3. 18] was derived and is only valid for steady states.

As for the kinetic parameters, the yield and maintenance coefficients may vary with the current state of the culture (culture conditions).

3. 4. Calculation of oxygen and carbon dioxide transfer rates

Gas-liquid mass transfer rates for oxygen and carbon dioxide can be calculated by measuring the flow of air into the bioreactor, and the concentrations of oxygen and carbon dioxide in the exhaust gas. By applying an inert gas balance, the flow of the exhaust gas need not to be measured:

$$OTR = F_G M_{O2} (x_{O2}^{in} - x_{O2}^{out} (1 - x_{O2}^{in} - x_{CO2}^{in})/(1 - x_{O2}^{out} - x_{CO2}^{out})))/(V_M V_L) \quad [3.\ 20]$$

$$CTR = F_G M_{CO2} (x_{CO2}^{out}(1 - x_{O2}^{in} - x_{CO2}^{in})/(1 - x_{O2}^{out} - x_{CO2}^{out}) - x_{CO2}^{in}))/(V_M V_L) \quad [3.\ 21]$$

with:

F_G	flow of air into the reactor (L h^{-1})
M	molar mass (g mol^{-1})
x_{O2}^{in}	molar fraction of O_2 in inlet gases (mol mol^{-1})
x_{O2}^{out}	molar fraction of O_2 in outlet gases (mol mol^{-1})
x_{CO2}^{in}	molar fraction of CO_2 in inlet gases (mol mol^{-1})
x_{CO2}^{out}	molar fraction of CO_2 in outlet gases (mol mol^{-1})
V_M	molar volume at standard temperature and pressure (STP) = 22.414 (L mol^{-1})
V_L	reactor working volume (L)

It should be stated here, that the transfer rates are not identical with the biological oxygen uptake rate OUR and the carbon dioxide evolution rate CPR, respectively. However, for oxygen because of its low solubility in water, the difference between OTR and OUR is negligible. The same applies for carbon dioxide at equilibrium if the dilution rates (convective flow) and the pH (formation of bicarbonate) are not too high. In this case, the specific oxygen uptake rate and the specific carbon dioxide evolution rate can be directly calculated from the transfer rates:

$$r_{CO2} = CTR / C_X \quad [3.\ 22]$$

$$r_{O2} = OTR / C_X \quad [3.\ 23]$$

3. 5. Special cultivation techniques

3. 5. 1. Wash-out cultivation

Wash-out of a continuous culture can be used to calculate maximum growth or substrate conversion rates.This is also a powerful technique in the study of microbial behaviour (Esener et al. 1981). In a wash-out cultivation, the dilution rate of a continuous culture, running in steady state, is increased to a value above the critical dilution rate.

If the dilution rate is greater than the maximum specific growth rate, and no inhibitory substrates or products are present, the mass balance for biomass can be integrated:

$$\ln (C_X / C_{X\,0}) = (\mu_{max} - D)\ t \qquad [3.\ 24]$$

with: $\mu = \mu_{max}$ and $r_S = r_{S\,max}$.

A plot of $\ln(C_X / C_{X\,0}) = f(t)$ results in a straight line and μ_{max} can be obtained from the slope. In the presence of inhibitory compounds or inhibitory substrate the mass balance can not be integrated, but the time course of μ and r_S can be calculated graphically by:

$$\mu = D + dC_X / (dt\ C_X) \qquad [3.\ 25]$$

$$r_S = (D\ (C_{S\,0} - C_S) - dC_S / dt) / C_X \qquad [3.\ 26]$$

3. 5. 2. A-stat or substrate ramp cultivation

It is usually difficult to identify the substrate affinity because a batch experiment is not sensitive enough, and in continuous cultivations the substrate concentration is usually too low to be measured by off-line analytics. If the dilution rate is increased steadily in a continuous cultivation with a slow pace from a value below the maximum growth rate to a value above the maximum growth rate (A-stat, D-ramp), the K_S-sensitive range is expanded, and, considering the kinetic equation is known, K_S can be identified from a data fit with greater accuracy. The method is proposed and published by Paalme et al. (1995 & 1997) and was also be used by Kies (1998) in his PhD-work to identify quinoline conversion kinetics.

Compared with a serious of steady states, the A-stat is not so time-consuming, especially at higher acceleration factors. However, as was shown by Paalme et al. (1995 & 1997), the acceleration factor must be selected carefully. If the time constant of the acceleration is higher than the metabolic time constants, the results are influenced by the process dynamics. At sufficiently low acceleration factors, quasi steady state conditions usually achieved with an A-stat.

For more than one feed streams into the reactor, the overall acceleration factor is the sum of the individual factors. In all A-stat cultivations, a smooth increase in the dilution rate was started according to the following equation:

$$D = D_0 + a_{all}\ t \qquad [3.\ 27]$$

where:
- D dilution rate (h^{-1})
- D_0 initial dilution rate (h^{-1})
- a_{all} overall acceleration rate (h^{-2})
- t experimental time (h)

To estimate the specific rates during A-stat cultivations, the equations [3. 25] and [3. 26] obtained for wash-out cultivations can be used, but here the dilution rate D is a function of time.

3. 6. Carbon balance (carbon recovery)

Carbon balances are used for checking completeness of degradation. If the carbon balance is not fulfilled, the formation of an unknown metabolic intermediates must be considered. For continuous cultivations at steady state, the carbon balance can be calculated by comparing all in and outgoing flows of carbon containing compounds:

$$C_{rec} = (Da_{CX}C_X + a_{CO2}CTR + D\sum_i (a_{Ci}C_i) / (Da_{CS}C_{S0}) \qquad [3.\ 28]$$

with: a_{Ci} mass fraction of carbon in compound i (g g^{-1})

Here, the substrate concentration was considered to be zero and convective flow of carbon dioxide was neglected (see above). The term $\sum(a_{Ci} C_i)$ expresses known reaction products and can be used if appropriate.

For batch experiments, the carbon balance can either be expressed as a differential balance or as an integral balance. The differential balance is very sensitive to small transient changes but also to data scatter. In biological systems, data scatter, especially for biomass data, is often too high to apply differential carbon balances. The integral balance compares the current situation of the culture state with the initial state. Small alterations of the carbon balance at a certain time can usually not be detected, but it is relatively reliable to detect accumulation of unknown substances if their amount exceeds 5%. In this work, only integral carbon balances will be used for batch experiments:

$$C_{rec} = \frac{V_{L\,(t)}\,(a_{CX}\,C_X + a_{CS}\,C_S + a_{CCO2}\int CTR\,dt + \sum(a_{Ci}\,C_i)_{(t)} + \sum(V_{(pi)}\,(a_{CX}\,C_{X\,(pi)} + a_{CS}\,C_{S\,(pi)} + \sum(a_{Ci}\,C_i)_{(t)}))}{V_{L\,(t=0)}\,(a_{CX}\,C_{X\,(t=0)} + a_{CS}\,C_{S\,(t=0)}} \qquad [3.\ 29]$$

In this work, because of the small reactor working volume, the change of reactor volume and the amount of compounds taken out of the reactor by frequent sampling can not be neglected. This is expressed by the second term in the equation.

For wash-out cultivations, carbon recovery can be expressed by the following equation:

$$C_{rec} = \frac{a_{CS}\, C_{S\,(t)} + a_{CX}\, C_{X\,(t)} + \sum(a_{Ci} C_i)_{(t)} + a_{CS}\, D \int C_{S(t)}\, dt + a_{CX}\, D \int C_{X(t)}\, dt + a_{C\,CO2} \int CTR\, dt + \sum(a_{Ci} D \int C_i\, dt}{a_{CS}\, C_{S\,(t=0)} + a_{CX}\, C_{X\,(t=0)} + a_{CS}\, D\, C_{S\,0}\,(t-t_0)} \qquad [3.30]$$

Here the C_{rec} is expressed as the amount of carbon present at time t in the rector plus the amount of carbon which is removed from the rector between t_0 and t related to the amount of carbon present at t_0 in the reactor plus the amount of carbon fed into the reactor between t_0 and t.

The integral balance of carbon in the culture was also used to calculate carbon recovery of A-stat cultivations. However, the dilution rate is not constant and is calculated based on D_0, a-factor, and time. The carbon recovery can be represented by:

$$C_{rec} = \frac{a_{CS}\, C_{S\,(t)} + a_{CX}\, C_{X\,(t)} + \sum(a_{Ci}\, C_i)_{(t)} + a_{CS}\,(D_0 \int C_{S\,(t)}\, dt + a_{all} \int C_{S\,(t)}\, t\, dt + a_{CX}\,(D_0 \int C_{X\,(t)} dt +}{a_{CS}\, C_{S\,(t=0)} + a_{CX}\, C_{X\,(t=0)} + (a_{CS}\, C_V\, (F_{0\,S}\, t + a_S\, V_L\, t^2/2)/V_L)} +$$

$$\frac{a_{all} \int C_{X\,(t)}\, t\, dt) + a_{C\,CO2} \int CTR\, dt + \sum(a_{Ci}\,(D_0 \int C_{i\,(t)}\, dt + a_{all} \int C_{i\,(t)}\, t\, dt))}{a_{CS}\, C_{S\,(t=0)} + a_{CX}\, C_{X\,(t=0)} + (a_{CS}\, C_V\, (F_{0\,S}\, t + a_S\, V_L\, t^2/2)/V_L)} \qquad [3.31]$$

Where:
- a_S a-factor of the studied substrate
- C_V feed concentration of the studied substrate
- $F_{0\,S}$ initial flow of the studied substrate.

It was also taken into account, that all terms dealing with the substrate must be repeated according to number of the carbon sources used, whether this equation was used for the different mixtures.

4. RESULTS AND DISCUSSION

Many of the substances considered as pollutants in the environment are carbon and energy sources for microbial growth. Of them, three compounds possessing different properties were selected to investigate simultaneous biological degradation in this work. Phenol is a substance classified as toxic by existing environmental regulations and can represent wastes of agricultural and industrial origin. Also, benzoate is degraded by the same metabolic pathway, but less toxic or inhibitory and widely distributed as one of the aromatic contaminates. The easy degradable compound is sodium acetate. It was selected because it is a minor toxic aliphatic substrate which can represent wastes of urban or industrial origin.

In general, performance of a microbial strain or a microbial community to degrade various substrates is determined by the kinetics properties towards each possible substrate at the given conditions. Specific emphasis was therefore directed to the possible non-constancy of kinetic parameters. Although some work has been done in this direction in recent years, dynamic experiments were not performed out of a controlled culture history. One of them, main aims of this work will be to fill this gap in the ongoing discussions about this topic.

4. 1. Substrate biodegradation under chemostat conditions

4. 1. 1. Chemostat cultivation of the single substrates

Cultivation of these pollutants under substrate-limiting conditions to estimate stoichiometric parameters is the main purpose for this chapter. Therefore, phenol, benzoate and sodium acetate were cultivated individually in continuous culture (chemostat system) by using a pure strain of *Burkholderia (Pseudomonas) cepacia* G4.Steady states were established and investigated with each of the studied substrates for dilution rates ranging from 0 to ≈ 0.30 h^{-1}. This range was based on data recorded by Schröder et al. (1997), who determined the maximum dilution rate (equals maximum specific growth rate) for *B. cepacia* G4 on phenol to be 0.30 h^{-1}. For benzoate and sodium acetate, initially the same dilution rate range was investigated. However, a wash out experiment with sodium acetate at D = 0.48 h^{-1}, carried out later in this work to determine kinetic parameters, did not lead to a wash out, but approached a steady state (see chapter 4. 2. 1. 4. 2). Although this steady state was only observed for 1.2 residence times, the data were added here, because they fit perfectly to the other steady states. For all other steady state, five hydrodynamic residence times were waited before the first sample was taken. After that, from three to six samples were taken and the average value used for further calculation. From the recorded data, the specific substrate consumption rate (r_S), the carbon dioxide production rate (r_{CO2}), and the specific rate of oxygen uptake (r_{O2}) were calculated for each substrate.

The experimental results, as well as calculated values, are shown in Table (4.1). The calculated specific rates for substrate consumption were plotted versus the dilution rate in Fig. (4.1).

Table 4. 1. Experimental and calculated data for individual cultivation of phenol, benzoate and sodium acetate under substrate limiting conditions (chemostat operation).

Substrate	D h^{-1}	C_X g L^{-1}	C_{S0} g L^{-1}	OTR $gL^{-1}h^{-1}$	CTR $gL^{-1}h^{-1}$	C_{rec} g g^{-1}	r_S $gg^{-1}h^{-1}$	r_{CO2} $gg^{-1}h^{-1}$	r_{O2} $gg^{-1}h^{-1}$	$Y_{X/S}$ g g^{-1}	$Y_{X/CO2}$ g g^{-1}	$Y_{X/O2}$ g g^{-1}	$Y_{CO2/S}$ g g^{-1}
Phenol	0.051	1.284	2.162	0.174	0.174	0.914	0.085	0.135	0.135	0.594	0.375	0.375	1.583
	0.102	1.590	2.163	0.393	0.336	0.973	0.139	0.211	0.247	0.735	0.485	0.414	1.516
	0.220	1.610	2.123	0.752	0.662	0.952	0.290	0.411	0.467	0.758	0.534	0.471	1.419
Benzoate	0.052	1.223	1.873	0.172	0.157	1.072	0.079	0.128	0.141	0.653	0.405	0.369	1.626
	0.104	1.320	1.873	0.275	0.299	1.066	0.148	0.226	0.209	0.705	0.460	0.498	1.527
	0.223	1.457	2.017	0.703	0.718	1.105	0.309	0.493	0.483	0.722	0.453	0.462	1.595
	0.257	1.358	1.915	0.674	0.696	1.025	0.362	0.513	0.497	0.709	0.501	0.517	1.416
Sodium acetate	0.048	1.079	3.802	0.129	0.100	0.943	0.170	0.092	0.120	0.284	0.522	0.404	0.544
	0.094	1.249	3.771	0.194	0.199	1.031	0.285	0.159	0.156	0.331	0.592	0.606	0.559
	0.205	1.073	2.950	0.302	0.369	1.128	0.564	0.344	0.282	0.364	0.597	0.728	0.610
	0.238	1.176	2.993	0.380	0.445	1.188	0.605	0.379	0.323	0.393	0.628	0.736	0.626
	0.480	1.205	3.240	0.658	0.805	1.055	1.290	0.668	0.546	0.372	0.719	0.878	0.517

F_G = 162 L h^{-1} (STP), V_L = 2.52 L.

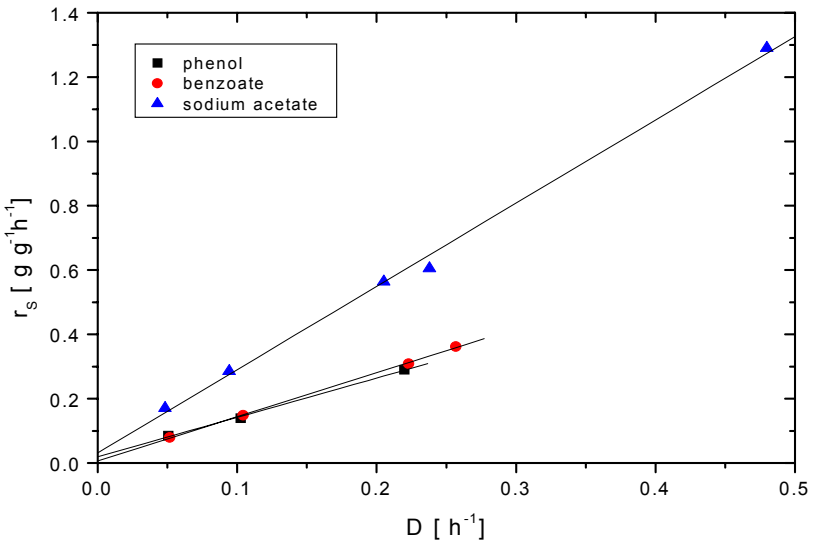

Fig. 4. 1. Specific substrate consumption rates of phenol, benzoate and sodium acetate as function of the dilution rate under steady state conditions.

It is obvious that the specific consumption rates for both phenol and benzoate are approximately identical at the studied dilution rates. On the other hand, the specific conversion rates of sodium acetate are twice as high compared with phenol and benzoate at the same dilution rates.

Because phenol and benzoate are metabolized via the same degradation sequence with exception of the first step, the conversion to catechol, resulting in one mole each of pyruvate and acetaldehyde from conversion of one mole of substrate, identical yield coefficients should be expected if expressed in molar conversions. Fig. (4. 2) shows the specific conversion rates recalculated as molar rates. The data for benzoate are slightly lower compared with those for phenol, but the difference is not very significant. The molar based maintenance coefficient for benzoate is 3 times lower compared with phenol. This may be caused by an additional burden placed upon the cell by the toxic compound phenol, which is known to attack the cell membrane (Keweloh et al. 1989 &1990).

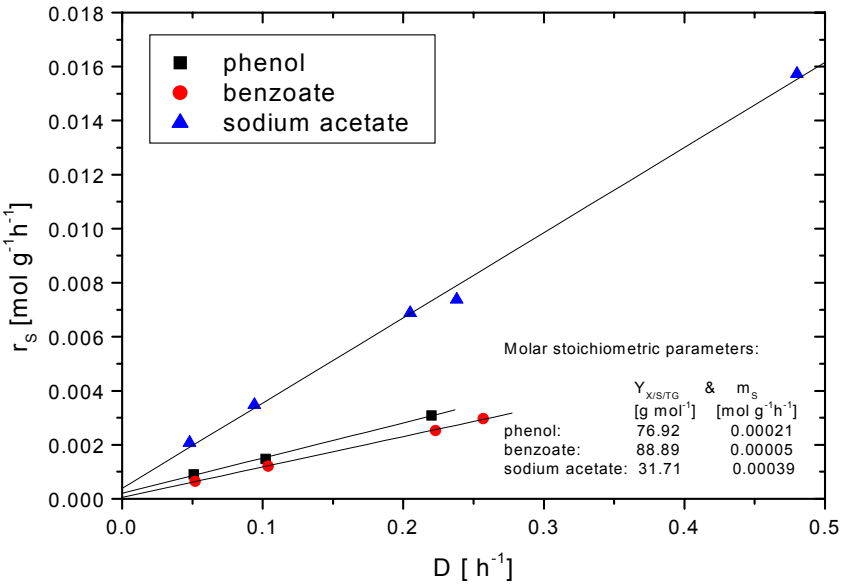

Fig. 4. 2. Molar specific consumption rates for phenol, benzoate and sodium acetate as function of the dilution rate under steady state conditions.

From the slopes of the curves in Fig. (4. 1), the biomass yield to consumed substrate ("True Growth", $Y_{X/S/TG}$) and maintenance coefficients (m_S) were calculated using linear regression techniques. The estimated values of $Y_{X/S/TG}$ and m_S for phenol degradation are 0.82 g g^{-1} and 0.020 g g^{-1} h^{-1}, respectively, with regression degree of R = 0.999. It means, that mineralization of one gram phenol leads to 0.82 gram biomass.

Steady state phenol degradation by *B. cepacia* G4 was also investigated by Schröder et al. (1997). Data obtained by Schröder et al. (1997) show that phenol degradation can be described by the same set of stoichiometric parameters under substrate limitation and under high substrate concentrations in the inhibitory branch. The authors estimated the stoichiometric parameters to $Y_{X/S/TG}$ = 0.78 g g^{-1} and m_S = 0.013 g g^{-1} h^{-1}, respectively.

Growth analysis of this strain using phenol as a sole source of carbon resulted in $Y_{X/S}$ = 0.76 g g^{-1} and m_S = 0.028 g g^{-1} h^{-1} in continuous culture (Solomon et al. 1994). It is important to mention that a lower value of 0.52 g g^{-1} was obtained by a pure culture of *P. putida* in continuous culture (Şeker et al. 1997). On the other hand, higher stoichiometric parameters of $Y_{X/S}$ = 0.94 g g^{-1} and m_S = 0.050 g g^{-1} h^{-1} were obtained by Fischer (1989).

A comparison between the data for phenol cultivation of Schröder et al. (1997), and this work shows good agreement (Fig. 4.3), indicating that the strain has not changed and that the data of Schröder et al. (1997) can be used for this work. In addition, the data for the specific benzoate conversion rates are also plotted in Fig. (4. 3).

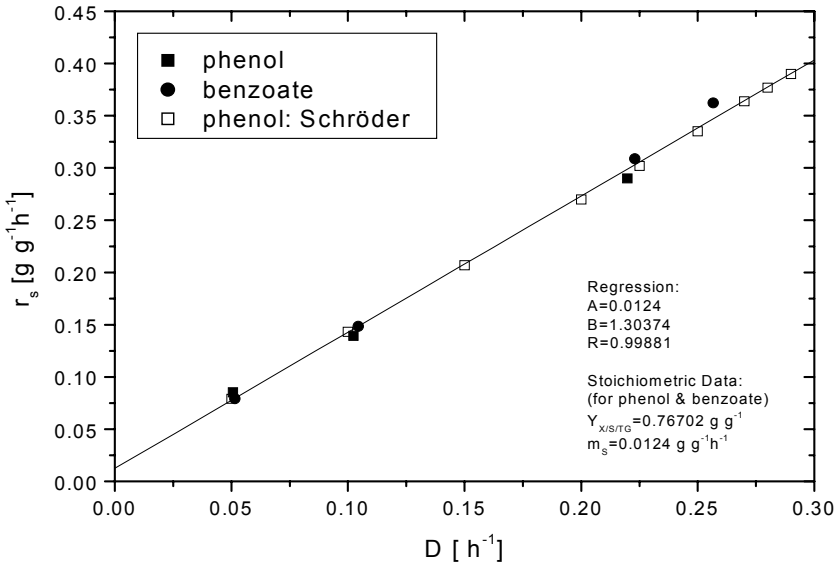

Fig. 4. 3. A comparison between specific consumption rates of phenol calculated by Schröder et al. (1997), and in this work, and of benzoate from this work.

A plot of r_S for benzoate versus the dilution rate D resulted in a straight line. From the slope in Fig. (4. 1), $Y_{X/S/TG}$ = 0.73 g g^{-1} and m_S = 0.0064 g g^{-1} h^{-1} (R =1) for benzoate degradation was calculated.

During growth on benzoate, a yellow product showed up in the bioreactor, indicating a conversion of catechol via catechol-2-3-dioxygenase (*meta* route) to 2-hydroxymuconic semialdehyde. This was also observed by Ampe and Lindley (1995) and Smith (1990).

The growth behaviour of *Alcaligenes eutrophus* using various concentrations of benzoate was investigated by Ampe and Lindley (1996). The authors determined the stoichiometric parameters in a benzoate-limited chemostat. For this organism, a higher biomass yield of $Y_{X/S}$ = 0.87 g g^{-1}, and a maintenance coefficient of m_S = 0.018 g g^{-1} h^{-1} were obtained.

The carbon recovery data for phenol and benzoate are given in Table (4. 1). For benzoate, the carbon recovery is fulfilled, indicating that the conversion of benzoate to biomass and carbon dioxide is complete. For phenol, the carbon recovery for D = 0.10 h^{-1} is 0.97 g g^{-1}, which can also be considered as fulfilled. For the other two dilution rates on phenol, the carbon recovery data are slightly lower (0.91 and 0.95, respectively), so these data may indicate formation of an intermediate. But in combination with the phenol recovery data of Schröder et al. (1997), the carbon recovery for phenol can also be considered as fulfilled.

The specific conversion rates of sodium acetate, as already mentioned, were considerably higher than those for phenol and benzoate at the same dilution rate. Additionally, steady states up to a dilution rate of 0.48 h^{-1} were obtained, indicating that the maximum growth rate on acetate is quite higher than on phenol (μ_{max}=0.30 h^{-1} according to Schröder et al. 1997).

From the slope in Fig. (4. 1), biomass yield and maintenance coefficients for sodium acetate were calculated to be $Y_{X/STG}$ = 0.39 g g^{-1} and m_S = 0.031 g g^{-1} h^{-1}, respectively, with regression degree of R = 0.998. The same yield was estimated by Ampe and Lindly (1995) using *Alcaligenes eutrophus* 335. Hernandez and Johnson (1967) estimated the biomass yield of both *Candida utilis* and *Pseudomonas fluorescens* grown on acetate to 0.36 and 0.28 g g^{-1} at limiting substrate conditions, respectively. The data obtained by Holms (1987) are also in good agreement with the data of this work. The author determined the yield of *Escherichia coli* growing on acetate as a sole source of carbon and energy as 0.40 g g^{-1}.

As for phenol and benzoate, carbon recovery data for sodium acetate are also fulfilled (see Table 4. 1). The same result was obtained by Holms (1987). It means that all substrates were converted completely to biomass and carbon dioxide at steady states under substrate limiting conditions.

According to the theory of Kovárová-Kovar and Egli (1998), the stoichiometric and kinetic parameters are variable and subject to the cultivation conditions. At carbon limited growth, the microorganisms use their maximum possible yield for biomass formation. The estimated values represent therefore the upper limit of the biomass yield.

Fig. (4. 4) shows the specific carbon dioxide production rate r_{CO2} of the studied substrates as a function of the dilution rate D.

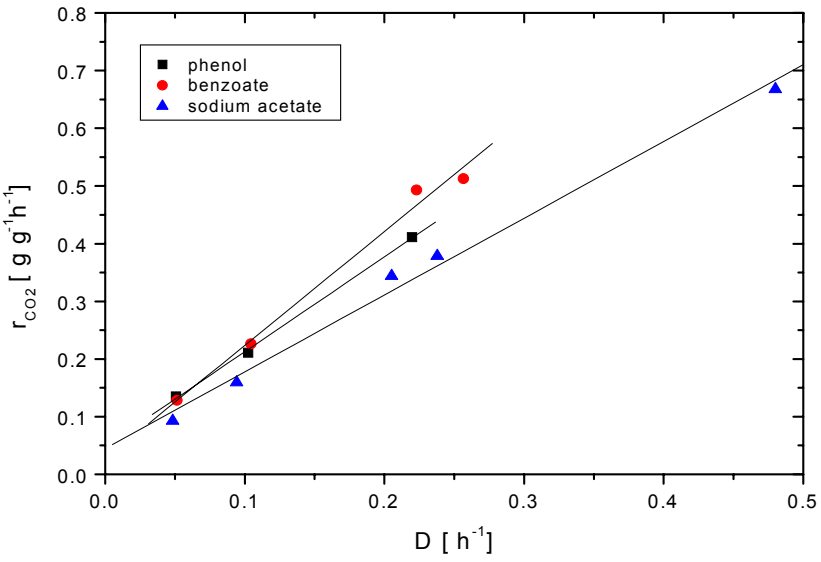

Fig. 4. 4. Specific carbon dioxide production rate versus dilution rate for the studied substrates under steady state conditions.

For all three substrates, straight lines were obtained, but in contrast to r_S the phenol and benzoate plots do not seem to fall together. The biomass to carbon dioxide yield and maintenance parameters obtained by linear regression, are:

phenol: $Y_{X/CO2/TG} = 0.61$ g g^{-1}, $m_{CO2} = 0.048$ g g^{-1} h^{-1}, R = 0.999
benzoate: $Y_{X/CO2/TG} = 0.51$ g g^{-1}, $m_{CO2} = 0.026$ g g^{-1} h^{-1}, R = 0.995
sodium acetate: $Y_{X/CO2/TG} = 0.75$ g g^{-1}, $m_{CO2} = 0.045$ g g^{-1} h^{-1}, R = 0.996

The deviation between phenol and benzoate may be caused by data scatter, because only three steady states for phenol were investigated. Therefore, the data of Schröder et al. (1997) were included in this comparison, and the rates were recalculated as:

phenol: $Y_{X/CO2/TG} = 0.57$ g g^{-1}, $m_{CO2} = 0.046$ g g^{-1} h^{-1}, R = 0.999

To obtain the carbon dioxide to substrate yield coefficients, $Y_{CO2/S}$, the data of the specific carbon dioxide production rates as function of the specific substrate consumption rates for all substrates are plotted in Fig. (4. 5). Because carbon dioxide can not be produced without consumption of substrate in steady state, the data were fitted to straight lines passing through the origin. From the results represented in Fig. (4. 5) it was found that phenol and benzoate plots can be expressed by the same line. From the slopes, the parameters of carbon dioxide yield of all substrates are:

phenol: $Y_{CO2/S/TG}$ = 1.48 g g-1
benzoate: $Y_{CO2/S/TG}$ = 1.48 g g^{-1}
sodium acetate: $Y_{CO2/S/TG}$ = 0.55 g g^{-1}

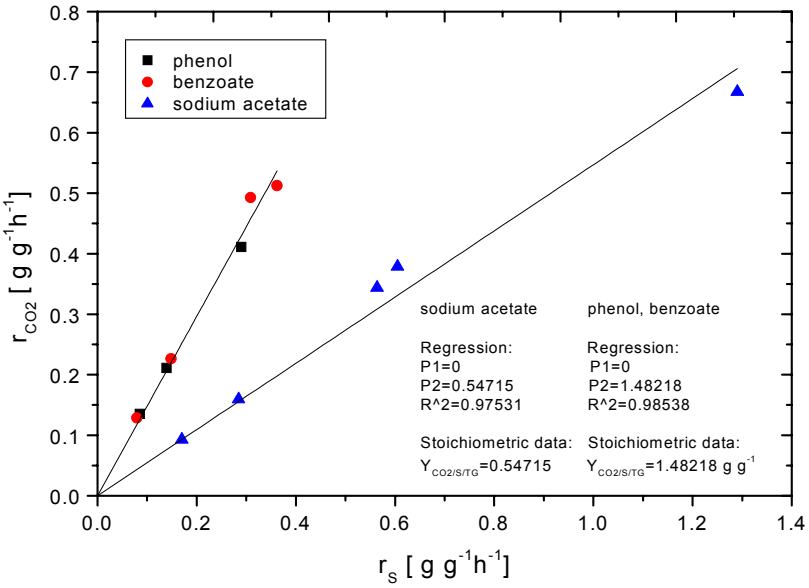

Fig. 4. 5. Specific carbon dioxide production rates versus specific substrate consumption rates of phenol, benzoate and sodium acetate under steady state conditions.

Fig. (4. 6) shows the specific oxygen uptake rate r_{O2} of the studied substrates as function of the dilution rate D. Straight lines for all three substrates were obtained with comparable data for phenol and benzoate.

The calculated yield and maintenance coefficients are:

phenol:	$Y_{X/O2/TG}$ = 0.51 g g^{-1} ,	m_{O2} = 0.041 g g^{-1} h^{-1} ,	R = 0.999
benzoate:	$Y_{X/O2/TG}$ = 0.53 g g^{-1} ,	m_{O2} = 0.033 g g^{-1} h^{-1} ,	R = 0.991
sodium acetate:	$Y_{X/O2/TG}$ = 1.00 g g^{-1} ,	m_{O2} = 0.072 g g^{-1} h^{-1} ,	R = 0.995

In view of the experimental data obtained, *B. cepacia* G4 was cultivated successfully using phenol, benzoate and sodium acetate as sole sources of carbon and energy at different dilution rates under substrate limiting conditions. The results for acetate also show the independency of steady states on the substrate concentration in the medium feed, which is in accordance with the chemostat theory (Herbert et al. 1956).

According to the linear relationship between specific rates and dilution rate, the Pirt equation can be applied successfully for all substrates.

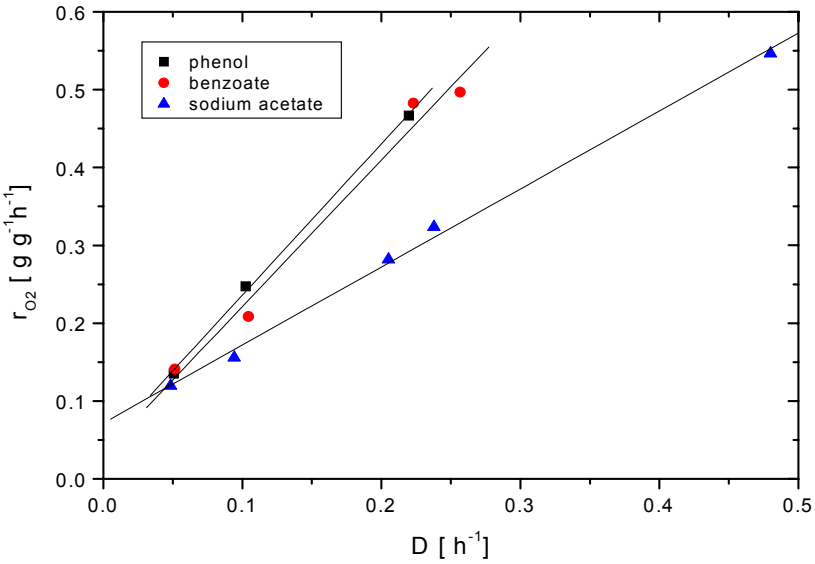

Fig. 4. 6. Specific oxygen uptake rates of phenol, benzoate and sodium acetate versus dilution rates under steady state conditions.

It should be mentioned, that the specific consumption rate of all substrates is depending only upon the dilution rate, which equals the specific growth rate under steady state conditions. Consequently, the specific substrate consumption rate is not influenced by a change in the substrate feed concentration (C_{S0}).

The carbon recovery data for benzoate and sodium acetate are fulfilled, indicating that the conversion to biomass and carbon dioxide is complete. For phenol, the mean value of the carbon recovery is slightly lower. This may indicates formation of an intermediate, but in combination with the phenol recovery data of Schröder et al. (1997), the carbon recovery for phenol can also be considered as fulfilled.

Due to its higher specific conversion rates, cultivation of sodium acetate led to lower yields of biomass and carbon dioxide compared with phenol and benzoate. Holms (1987) reported that 0.55 gram of sodium acetate are converted by *Escherichia coli* to carbon dioxide compared with 0.57 gram in this work.

Appearance of a yellow colouration in the reactor during cultivation of benzoate indicates formation of 2-hydroxymuconic semialdehyde (2-HMS). This intermediate is characterized by an intense yellow colouration. Such phenomena was not observed during phenol cultivation although the same metabolic route is used. The difference can be caused by either a regulation change of the metabolic enzymes or by the use of different enzymes in the *meta* pathway(isoenzymes). However, in spite of the formation of 2-HMS, the carbon recovery of benzoate is fulfilled, showing that the amount of 2-HMS generated is negligible small.

4. 1. 2. Chemostat cultivation of the ternary mixture of substrates

One of the major problems of the biological degradation of environmental pollutants in the real world is the co-existence of theses compounds in mixtures with hazardous and toxic chemicals. In the natural ecosystems, the microorganisms grow under complex conditions. Therefore, occurrence of environmental pollutants in mixtures is an important problem, because removal or degradation of one component can be prevented or inhibited by an other compound in the mixture, and different conditions may be required to treat different compounds within the mixture.

Degradation of the selected substrates was already assessed individually to estimate stoichiometric parameters. Under similar conditions, the culture was grown on ternary mixture of the selected substrates to investigate their utilization pattern in the mixture (simultaneous or sequential) and, to confirm whether the stoichiometric behaviour of the mixture can be expressed by the data obtained from cultivation of the single substrates. In addition, dependence of the steady state on the concentration of the particular substrate in the medium feed (mixture composition) will be investigated.

In order to realise the aim mentioned above, steady states were established in the same range of dilution rates ($0 \approx 0.30\ h^{-1}$) applied with the single substrates. This range was selected to compare cultivation of the substrate mixture with the cultivation of the single substrates. Although a higher dilution rate of $D = 0.48\ h^{-1}$ was obtained on sodium acetate, the exposition time was not long enough to regard it as a true steady state. Therefore, the range of dilution rates was remained as stated.

Under chemostat conditions, the cultivation was performed with ternary mixtures of phenol, benzoate and sodium acetate at four dilution rates. For each dilution rate, five hydrodynamic residence times were waited before the first sample was taken. After that, from three to four samples were taken and the mean value was used for further calculations. From the recorded data, the specific substrate conversion rate (r_S), the specific carbon dioxide production rates (r_{CO2}), and the specific rates of oxygen uptake (r_{O2}) were calculated. The experimental data as well as calculated values for chemostat conditions are given in Table (4. 2).

Table 4. 2. Experimental and calculated data for ternary mixture of phenol, benzoate and sodium acetate under substrate limiting conditions (chemostat cultivation).

D	C_X	C_{S0} g L^{-1}			OTR	CTR	C_{rec}	r_S	$Y_{X/S}$	r_{CO2}	$Y_{X/CO2}$	r_{O2}	$Y_{X/O2}$	$Y_{CO2/S}$
h^{-1}	g L^{-1}	phen.	benz.	s.acet.	$gL^{-1}h^{-1}$	$gL^{-1}h^{-1}$	g g^{-1}	$gg^{-1}h^{-1}$	g g^{-1}	$gg^{-1}h^{-1}$	g g^{-1}	$gg^{-1}h^{-1}$	g g^{-1}	g g^{-1}
0.054	0.894	0.712	0.660	0.854	0.158	0.181	1.055	0.134	0.401	0.202	0.266	0.176	0.305	0.203
0.108	1.188	0.895	0.587	0.850	0.330	0.366	1.095	0.211	0.510	0.308	0.349	0.278	0.387	0.359
0.155	1.323	0.959	0.601	1.019	0.468	0.535	1.065	0.301	0.513	0.404	0.383	0.353	0.438	0.502
0.244	1.437	0.883	0.638	0.861	0.680	0.711	1.054	0.405	0.603	0.495	0.494	0.473	0.516	0.635

F_G = 162 L h^{-1} (STP), V_L = 2.52 L.

To obtain a biomass concentration of approximately 1.2 g L^{-1}, as in the cultivations using single substrates, the feed concentrations of the three substrates were reduced compared with the previous chemostat cultivations.

No substrate was detected in the reactor. It indicates that all substrates associated in the ternary mixture were completely and simultaneously utilized. Hence, no diauxic growth was observed. Because of the simultaneous utilization, the substrate concentrations are low (below detection limit). Inhibitory effects of the substrates can therefore be neglected under these conditions. A yellow colouration was not detected in the culture medium. If different enzymes or a different regulatory pattern is induced by phenol and benzoate, then probably intermediates of benzoate are metabolized by the "phenol induced route", which may explain the absence of yellow colour.

As is demonstrated in Fig. (4. 7), that the carbon recovery of the ternary mixture is fulfilled. It means, that conversion of all substrates to biomass and carbon dioxide is complete. A similar result was obtained by Müller et al. (1996). The authors found that carbon balance during cultivation of *Pseudomonas sp*. B13 FR1 SN45P on a substrate mixture of chloro- and methylsubstituted benzoic acids was also fulfilled under steady state conditions.

In general, it can be stated, that *B. cepacia* G4 is capable to metabolize phenol, benzoate and sodium acetate not only as sole sources of carbon, but also as a ternary mixture completely under substrate limitation.

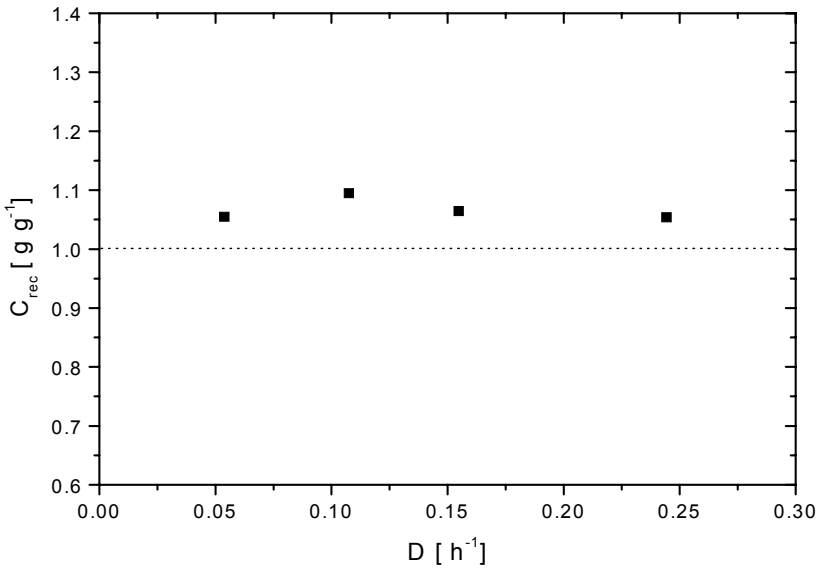

Fig. 4. 7. Carbon recovery for cultivation of substrate mixtures of phenol, benzoate and sodium acetate as a function of dilution rates under steady state conditions.

To compare degradation of the ternary mixture with the single substrates, the specific substrate consumption rate (r_S), and the biomass concentration were expressed as the sum of the three substrates:

$$r_S = D\,(C_{S0\,(p)} + C_{S0\,(b)} + C_{S0\,(s)}) / C_X \qquad [4.\ 1]$$

and:

$$C_X = Y_{X/S\,(p)}\,C_{S0\,(p)} + Y_{X/S\,(b)}\,C_{S0\,(b)} + Y_{X/S\,(s)}\,C_{S0\,(s)} \qquad [4.\ 2]$$

$$r_S = D\,(C_{S0\,(p)} + C_{S0\,(b)} + C_{S0\,(s)}) / (Y_{X/S\,(p)}\,C_{S0\,(p)} + Y_{X/S\,(b)}\,C_{S0\,(b)} + Y_{X/S\,(s)}\,C_{S0\,(s)}) \qquad [4.\ 3]$$

where:

$C_{S0\,(p)}$	initial feed concentration of phenol (g L^{-1})
$C_{S0\,(b)}$	initial feed concentration of benzoate (g L^{-1})
C_{S0} (s)	initial feed concentration of sodium acetate (g L^{-1})
C_X	biomass concentration (g L^{-1})
D	dilution rate (h^{-1})
r_S	specific substrate consumption rate of the mixture (g g^{-1} h^{-1})
$Y_{X/S\,(p)}$	biomass yield coefficient of phenol (g g^{-1})
$Y_{X/S\,(b)}$	biomass yield coefficient of benzoate (g g^{-1})
$Y_{X/S\,(s)}$	biomass yield coefficient of sodium acetate (g g^{-1})

In contrast to the single substrate cultivation, the initial feed concentrations in the mixture play an important role because due to the different yield coefficients, the biomass concentration is altered if the substrate composition changes in the feed. Therefore, approximately constant mean values of substrate concentrations in the mixture were used. According to equation [4. 1], the specific rates were calculated from the individual feed concentrations and the biomass concentration (Fig. 4. 8, a-c).

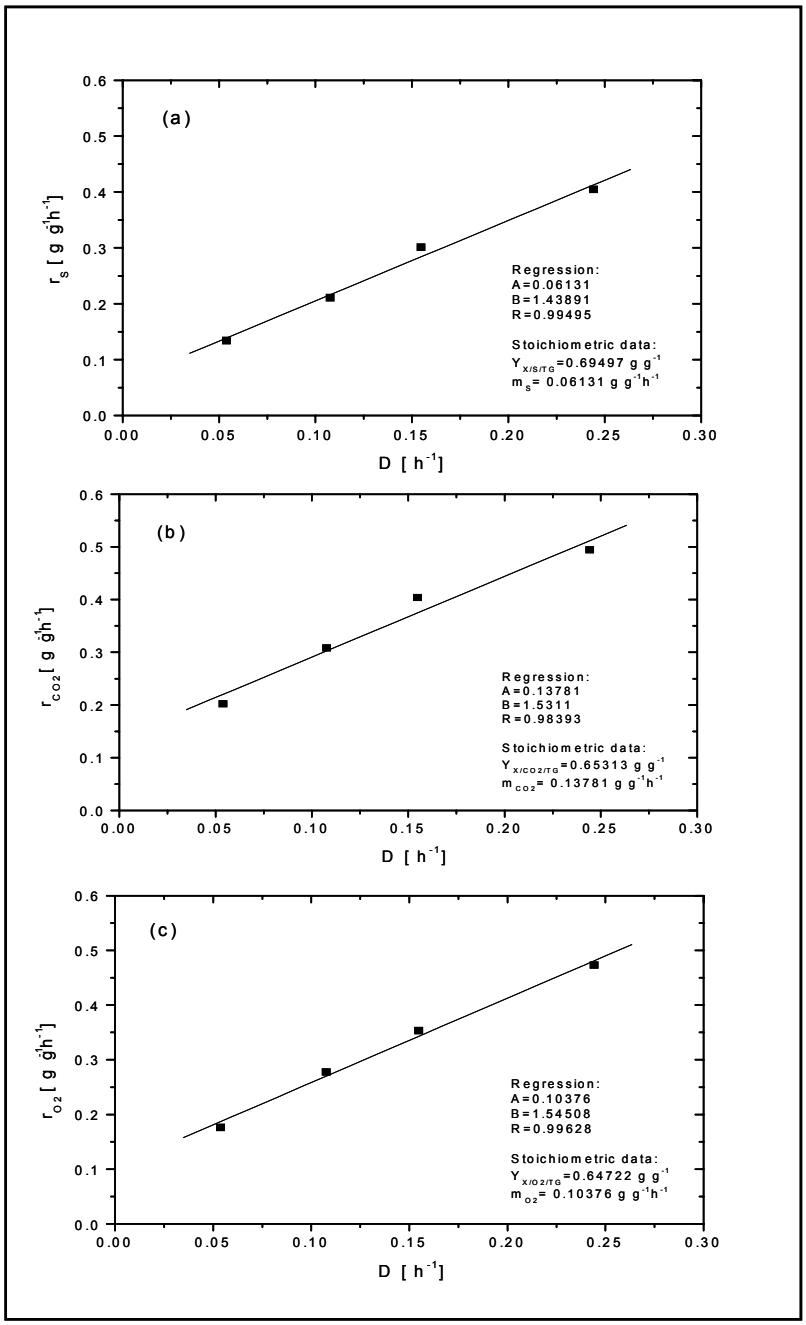

Fig. 4. 8. Linear regressions for the calculated specific rates obtained during cultivation of *B. cepacia* G4 on ternary mixture of phenol, benzoate and sodium acetate under steady state conditions.

Straight lines are obtained, showing the validity of the Pirt equation for the substrate mixtures. From the slopes, the yield and maintenance coefficients representing stoichiometric behaviour for this medium composition, were calculated as follows:

$$Y_{X/S/TG} = 0.69\ g\ g^{-1}, \quad m_S = 0.061\ g\ g^{-1}\ h^{-1}, \quad R = 0.995$$
$$Y_{X/CO2/TG} = 0.65\ g\ g^{-1}, \quad m_{CO2} = 0.138\ g\ g^{-1}\ h^{-1}, \quad R = 0.984$$
$$Y_{X/O2/TG} = 0.65\ g\ g^{-1}, \quad m_{O2} = 0.104\ g\ g^{-1}\ h^{-1}, \quad R = 0.996$$

Using equation [4. 2], the theoretical biomass concentrations were calculated with the yield and maintenance coefficients derived from single substrate conversions. Calculated and experimental data are compared in Table (4. 3). With exception of the lowest dilution rate ($D = 0.054\ h^{-1}$), good agreement was obtained. At lower dilution rates, the calculated biomass values are strongly influenced by the value of the maintenance coefficient, which can not be estimated with the same accuracy as the yield coefficient (unless steady states at very low dilution rates are investigated). This may be the reason for the higher deviation at this dilution rate. In addition, biomass percentages for each substrate were calculated.

Table 4. 3. A comparison between the calculated and the experimental biomass concentrations resulting from degradation of phenol, benzoate and sodium acetate in their mixture at different dilution rates.

D	phenol			benzoate			sodium acetate			total C_X	
	$Y_{X/S}$	C_X		$Y_{X/S}$	C_X		$Y_{X/S}$	C_X		calc.	exp.
h^{-1}	$g\ g^{-1}$	$g\ L^{-1}$	%	$g\ g^{-1}$	$g\ L^{-1}$	%	$g\ g^{-1}$	$g\ L^{-1}$	%	$g\ L^{-1}$	$g\ L^{-1}$
0.054	0.629	0.448	38.49	0.672	0.444	38.14	0.318	0.272	23.37	1.164	0.894
0.108	0.711	0.636	47.32	0.700	0.411	30.58	0.349	0.297	22.10	1.344	1.188
0.155	0.741	0.710	47.24	0.710	0.427	28.41	0.359	0.366	24.35	1.503	1.323
0.244	0.767	0.677	46.59	0.716	0.457	31.45	0.370	0.319	21.95	1.453	1.437

Beside its role as toxic component, phenol produces the highest amount of biomass of 44.9 % (mean value) during its conversion in the mixture, followed by 32.2 % for benzoate and only 22.9% for sodium acetate. These data high light to the role played by each substrate to produce biomass.

From the obtained results it can be concluded that the cultivation of a ternary substrate mixture of phenol, benzoate and sodium acetate can be expressed by the stoichiometric parameters obtained from the individually cultivations of these substrates. Like for the single substrate cultivations, Pirt´s equation can be successfully used to describe the stoichiometric parameters of the mixture. Under substrate limiting conditions, all substrates associated in the mixture were utilized completely. This means that the degradation efficiency of *B. cepacia* G4 has not changed when the culture was exposed to more complex conditions by using a ternary mixture of substrates, one of them is toxic. Moreover, it is interesting to mention that all substrates were utilized simultaneously without any detectable inhibitory effect. Like for the cultivation of the single substrates, carbon recovery data are fulfilled.

In general, it can be stated that the dependence of the steady states on the concentration of the particular substrate in the medium feed (mixture composition) does not contradict with the chemostat theory stated by Herbert et al. (1956). For a mixed substrate, not only the total substrate concentration but also the composition in the feed becomes relevant.

4. 2. Substrate biodegradation under dynamic conditions

Biological degradation of the selected substrates was investigated under steady state conditions. Steady state conditions are well suited to estimate stoichiometric parameters. Under substrate limiting conditions, maximum values of the yield are obtained. For estimation of kinetic parameters, dynamic conditions are usually necessary. The aim of this chapter is to investigate biological degradation of the single substrates as well as of their mixture, and to estimate kinetic parameters under various growth conditions.

Microbial growth kinetics are usually characterized by the maximum specific growth rate, μ_{max}, and the maximum specific substrate conversion rate, r_{Smax}, respectively, and by the substrate affinity K_S. In case of inhibitory influences by the substrate or reaction products, further parameters K_i are necessary to be taken into account (Müller and Babel, 1995).

Estimation of the maximum specific rates by chemostat cultivations is a time consuming process, a step-wise approach to the critical dilution rate. To avoid wash-out, the steps must be small in the vicinity of the critical dilution rate. Maximum rates are usually estimated using substrate surplus conditions (batch cultivation) or a wash-out experiment out of a continuous culture. However, in case of inhibitory substrates or products, maximum rates are often not obtainable by batch or wash-out cultivations because the inhibitory effect can reduce conversion rates even at low concentrations. Here, A-stat cultivation, which combines the advantages of substrate limitation and dynamic conditions, is an important technique. In an A-stat cultivation, the dilution rate is steadily increased with a constant acceleration rate (a-factor). Because of the slow transition from substrate limitation to substrate surplus, this technique is also well suited to estimate K_S, the substrate affinity constant (Müller et al. 1995). Batch cultivations are usually not sensitive in regard to this parameter.

All dynamic experiments used in this work were started out of a steady state, as “adjusted cultivations”. The adjusted cultivation guarantees identical initial conditions for each experiment (identical culture history), consequently the experiments can be repeated and compared under the same growth conditions. But it should be pointed out that the bacterial cells are not adapted to a sudden increase of the substrate concentration.

4. 2. 1. Cultivation of single substrates

4. 2. 1. 1. Biological degradation of phenol

4. 2. 1. 1. 1. Batch cultivation

All batches were carried out as "adjusted batches". Starting out of a chemostat at steady state, the feed pumps were halted and phenol was immediately added. After the batch cultivation, the feed pumps were restarted. The next batch was carried out after steady state conditions were re-obtained. In contrast to the "classical batch" cultivation, the adjusted batch experiments guarantee not only identical initial conditions but also100% active living cells, because resting cells or cell debris are washed out between the batches.

Four batch experiments were carried out using phenol as single substrate, and using different initial phenol concentrations. The "culture history" (dilution rate, phenol feed and steady state biomass concentration) as well as the initial phenol concentrations are given in Table (4. 4).

Table 4. 4. Culture history and initial phenol concentrations of the adjusted batch cultivations.

Experiments	Culture history			C_{S0}-batch g L^{-1}
	D-chem. h^{-1}	C_{S0}-chem. g L^{-1}	C_X-chem. g L^{-1}	
Sh-10	0.051	2.162	1.284	0.6427
Sh-12	0.051	2.162	1.284	0.7861
Sh-14	0.102	2.163	1.590	0.8954
Sh-15	0.220	2.123	1.610	0.9237

Fig. (4. 9) shows the batch (Sh-10) with the lowest initial phenol concentration (0.64 g L^{-1}). Plotted are dry weight of biomass, phenol concentration, and the oxygen and carbon dioxide transfer rates. The added phenol was rapidly consumed in 1.5 h. The biomass increased from 1.29 g L^{-1} to 1.38 g L^{-1} during this time period which corresponds to a yield of 0.14 g g^{-1}, considerable lower than at steady state. The time courses of OTR and CTR, which show a significant increase prior to the complete depletion of the substrate indicate substrate inhibition.

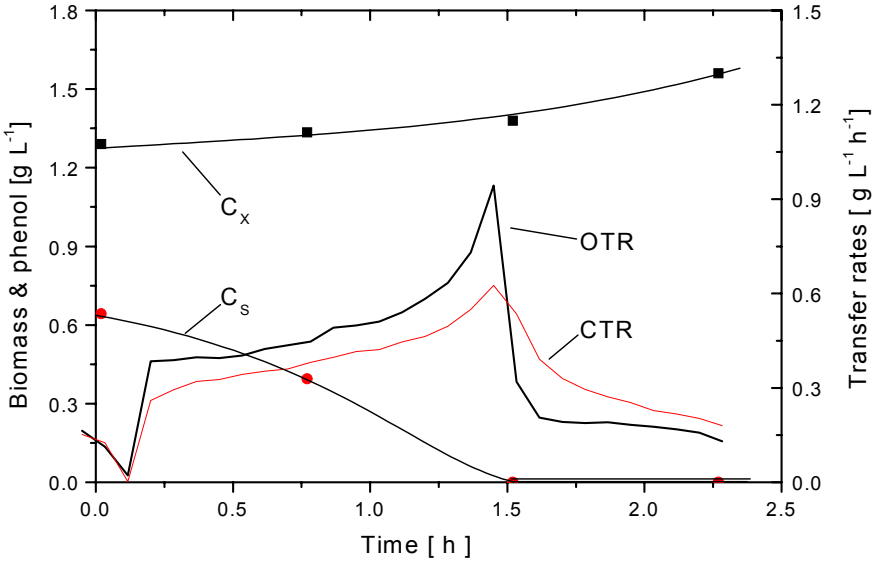

Fig. 4. 9. Experimental data obtained from batch cultivation Sh-10 on phenol.

The carbon recovery (not shown) constantly decreased and reached its minimum of 0.73 at the same time as the substrate is completely used. This means that about 27 % of the carbon of the added phenol is converted to unknown intermediates or otherwise missing. This does not correspond to the results of the steady states, where the carbon recovery was fulfilled.

After complete conversion of the substrate, OTR and CTR decreased rapidly, indicating a decrease in metabolic activity. Although the carbon recovery increased slightly after complete conversion of phenol, there seems to be a constant deviation remaining. On the other hand, the biomass increased further to 1.56 g L^{-1}, which increased the yield to 0.44 g g^{-1}. During the whole time of the experiment, the biomass yield remains considerably lower if compared with the chemostat.

The second batch (Sh-12), Fig. (4.10) was carried out with an initial phenol concentration of 0.79 g L^{-1} under the same conditions used for the previous batch. The substrate was complete consumed after 2 h. The biomass increased from 1.26 g L^{-1} to 1.55 g L^{-1}, and the OTR and CTR showed a similar time course as in the previous run. Here, no significant increase of the biomass after complete usage of the substrate was observed. The overall yield ($Y_{X/S}$ = 0.38 g g^{-1}) is as low as in the previous run.

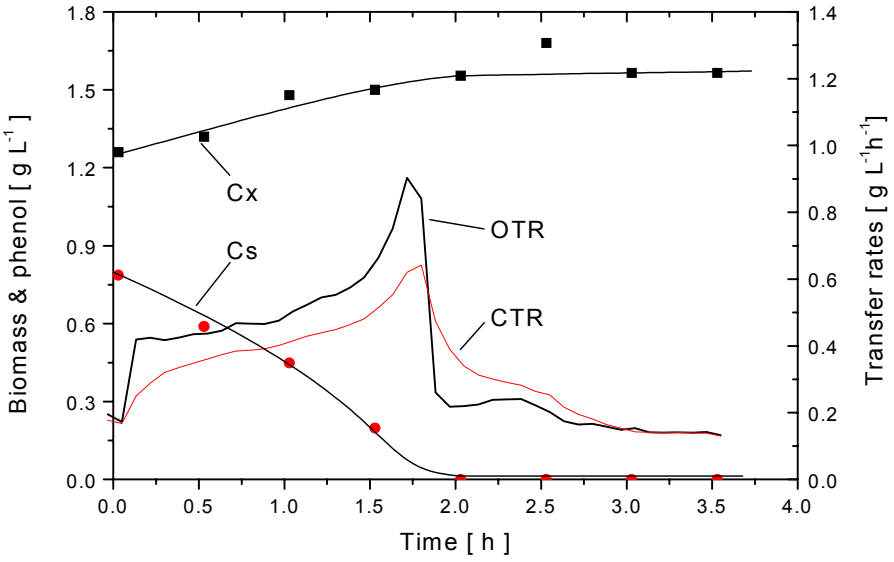

Fig. 4. 10. Experimental data obtained from batch cultivation Sh-12 on phenol.

The carbon recovery for this experiment (not shown) depicts a gradual decrease to a minimum of 0.78 at the time of total substrate depletion. After that, the C_{rec} increased slightly to 0.85 but seems to be constant after 2.5 h.

For the third batch run, Sh-14, the initial phenol concentration was increased further to 0.90 g L^{-1}. This time, the dilution rate prior to the experiment was doubled compared with both previous runs. But the phenol feed concentration was not altered. The experimental data are shown in Fig. (4.11). The substrate was mineralized completely after 2.4 h, resulting in a biomass increase from 1.62 g L^{-1} to 1.89 g L^{-1}. As in the preceding batch (Sh-12), no significant biomass increase was observed after complete conversion of the added phenol. The carbon recovery for this experiment is shown in Fig. (4. 12). It shows a slowly decrease before reaching its minimum of 0.82 at the time of total substrate depletion. After that, the carbon recovery remains constant.

The fourth batch (Sh-15) was carried out after a further increase of the dilution rate to a value close to the expected maximum dilution rate (D = 0.30 h^{-1}), given by Schröder et al. (1997). Approximately the same initial phenol concentration (0.92 g L^{-1}) as for the previous batch was supplied, but at a dilution rate of D = 0.22 h^{-1}. Although the initial biomass concentration was comparable to that of batch Sh-14, the substrate conversion took a considerable longer time, 6.5 h instead of 2.4 h, and the time courses of OTR and CTR show a more prominent effect of inhibition. During degradation of phenol, the overall biomass yield was 0.44, considerable lower compared with the steady state cultivation. The experimental data for this batch are plotted in Fig. (4. 13).

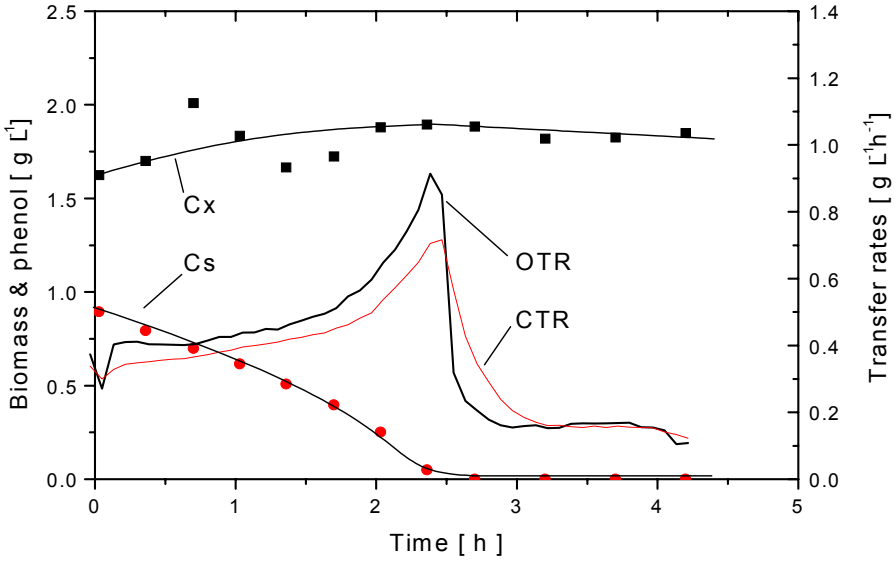

Fig. 4. 11. Experimental data obtained from batch cultivation Sh-14 on phenol.

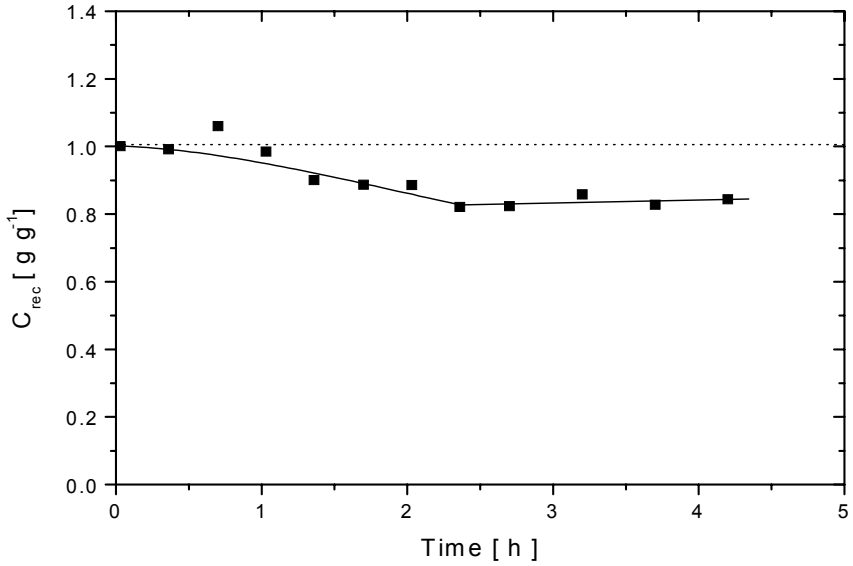

Fig. 4. 12. Carbon recovery for batch cultivation Sh-14 on phenol.

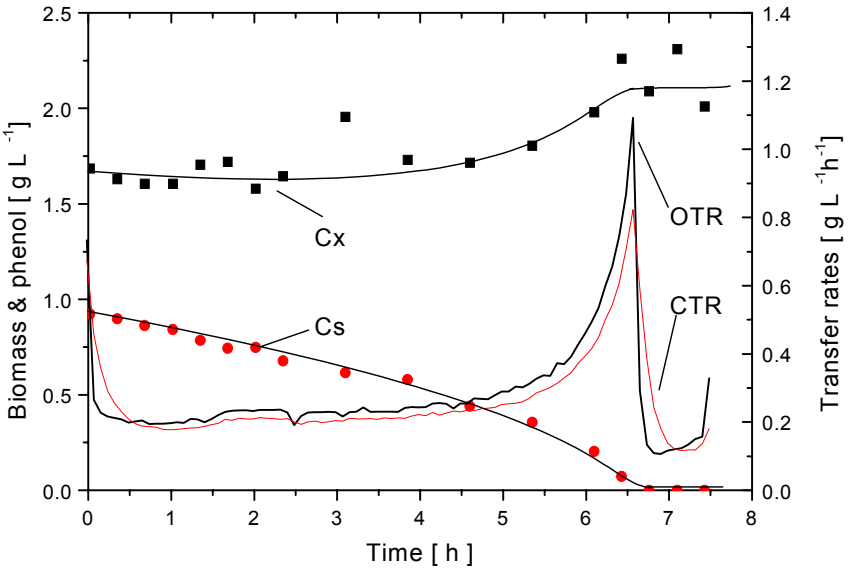

Fig. 4. 13. Experimental data obtained from batch cultivation Sh-15 on phenol.

Due to the better adaptation to higher metabolic rates, the carbon recovery (not shown) seems to be fulfilled in the first two hours. After that, slow decrease was observed and a minimum of 0.96 was reached at the time of total substrate depletion. After that, the carbon recovery seems to be constant. As for the previous runs, the carbon recovery data predict the formation of dead end metabolites. In all batch cultivations, the time courses of OTR and CTR reveal the influence of strong substrate inhibition.

In Fig. (4. 14), the time courses for substrate conversion of all four batches are compared. It demonstrates the significant difference of the two batches carried out at approximately identical initial concentrations of phenol, but originating from different dilution rates. It is not likely that this difference is solely the effect of the slightly higher initial substrate concentration of run Sh-15 (0.92 instead of 0.90 g L^{-1}). It seems that the "culture history" has a significant influence on the performance of the cells. Sh-15 was carried out of a dilution rate twice as high as for Sh-14, therefore the cells were adapted to a higher growth rate. In contrast, the initial growth rate of Sh-15 was considerable lower than that of Sh-14. This result contradicts in some way the hypothesis of Kovárová-Kovar and Egli (1998). Perhaps, at a growth rate close to the expected maximum specific growth rate, the microorganisms change their metabolic behaviour or produce small amount of inhibitors to reduce the substrate conversion rate to prevent accumulation of intermediates in the cell in case the flow through the catabolic chain exceeds the flow through the anabolic routes. The regulatory effect seems also to be connected with the carbon recovery. The lower metabolic rates of Sh-15, caused by a stronger inhibitory or regulatory effect also reduces the deviation of the carbon recovery.

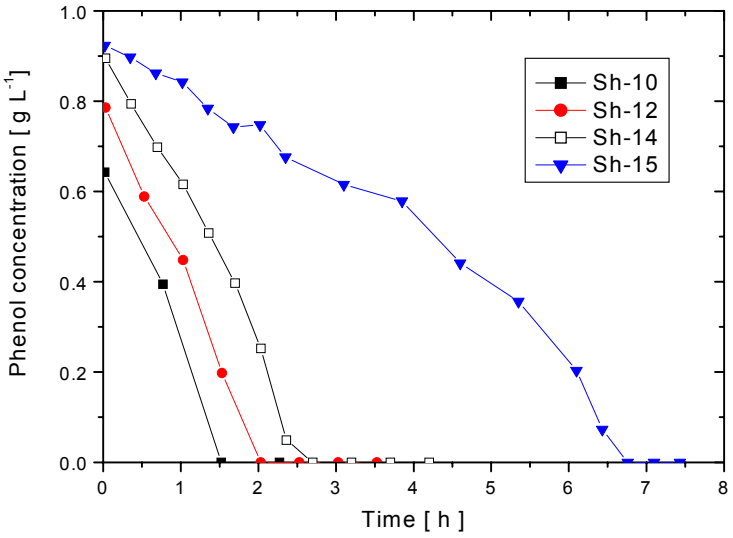

Fig. 4.14. A comparison between the time course for substrate conversion of the batch cultivations carried out on phenol.

In this context, it is interesting to compare the kinetics derived by Schröder et al. (1997) from unstable steady states with the batch experiments of this work. Schröder et al. (1997) proposed a Yano and Koga type kinetic equation:

$$r_S = r_{Smax}\ C_S / (K_S + C_S + C_S^3/K_i) \qquad [4.\ 4]$$

The parameters were given as: r_{Smax} = 0.409 g g^{-1} h^{-1}, K_S = 0.00055 g L^{-1}, and K_i = 0.089 g L^{-1}, respectively. The specific growth rate was expressed as:

$$\mu = Y_{X/STG}\ r_S - Y_{X/STG}\ m_S \qquad [4.\ 5]$$

with the parameters of $Y_{X/STG}$ = 0.78 g g^{-1}, and m_S = 0.013 g g^{-1} h^{-1}. With the kinetic equations and parameters given by Schröder et al., (1997), the mass balances for batch cultivation are:

$$dC_S / dt = - r_S\ C_X \qquad [4.\ 6]$$

and

$$dC_X / dt = \mu\ C_X\ ,\ \text{respectively.} \qquad [4.\ 7]$$

Simulations of progress curves were carried out using the MATLAB software platform. Fig. (4. 15) shows a comparison of the simulation results with the experimental data from this work for batch Sh-12 (Fig. 4. 15a) and batch Sh-15 (Fig. 4.15c). As is demonstrated in Fig. (4. 15a), phenol conversion of batch experiment Sh-12 is considerably faster than predicted by the Scröder kinetics. To overcome the reduced yield of the batch experiments compared with steady state cultivations, the simulation was repeated, but this time the biomass was not simulated. The experimental biomass data were interlinked with the ´spline´function of MATLAB and the so generated (quasi experimental) biomass data used for the numerical integration of equation [4. 6]. The mass balance for biomass equation [4. 7] was not used (Fig. 4. 15b). The result is the same, a much faster conversion of phenol than predicted by the steady state kinetics of Schröder et al. (1997). This applies for all batches, except for run Sh-15 (out of the high dilution rate). The simulation for this run is shown in Figures (4.15 c) and (4.15d). Simulation and experimental data correspond for more than 4 h. For the rest of the run, the simulation is faster than the batch. Here, the situation is reversed to the other batches.

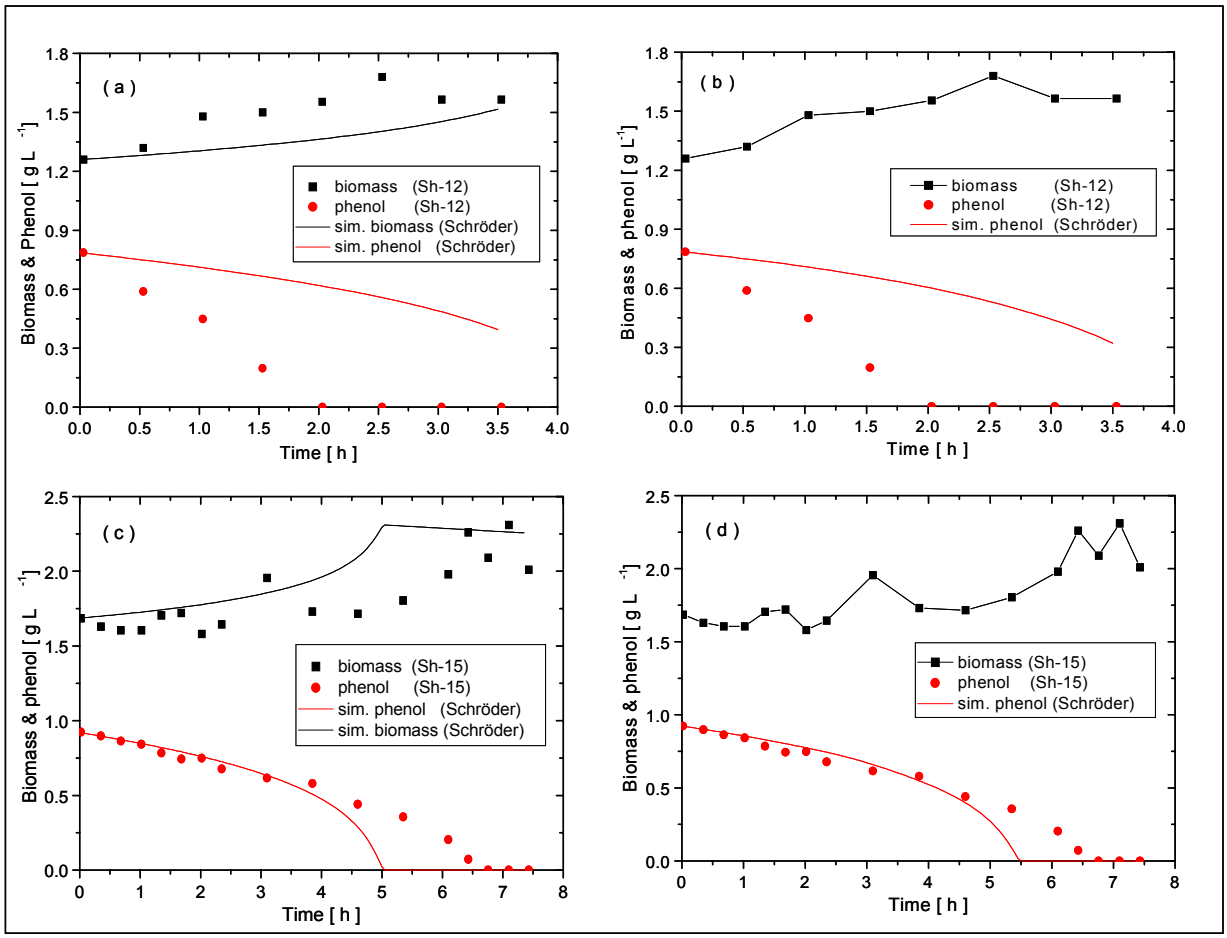

Fig. 4. 15. Comparison of experimental data (symbols) with the steady state kinetics of Schröder et al. 1997, (Lines) for two batch experiments Sh-12 and Sh-15.

It seems that the situation at the unstable steady states (substrate surplus, Schröder et al. 1997) roughly corresponds to the situation of run Sh-15. Perhaps, the higher substrate concentration in the preceding chemostat to this batch is the reason therefore. This means, not the specific growth rate, but the substrate concentration seems to have a regulatory function, which is in contrast to the hypothesis of Kovárová-Kovar and Egli (1998). According to their theory, the cells of Sh-15 are better adapted to high growth and conversion rates, because they had a higher growth rate in their "history". Therefor the initial growth rate should be higher than that of Sh-14. If this theory is valid, then the kinetics of phenol degradation by *B. cepacia* G4 is influenced by three time constants: 1) substrate inhibition of phenol, 2) growth rate of the "culture history", and 3) additional regulatory factor contradicting the effect of the growth rate. The "Schröder-kinetics", however, can not be used to describe the phenol batches.

Fig. (4.16) shows the calculated specific phenol conversion rates for all phenol batches via the phenol concentration. Because only two experimental points can be calculated, Sh-10 is not represented in this Figure. For comparison, the "Schröder-kinetics" is added. The relatively high scatter of r_S is caused by the biomass data. The data clearly show the influence of inhibition. All batches start at higher r_S-values than predicted by Schröder et al. (1997). In addition to the influence of the dilution rate of the preceding chemostat, it seems that there is also an influence of the initial phenol concentration: the lower the initial phenol concentration, the higher the specific phenol conversion rate. From the experimental results, it can be seen that the maximum specific phenol consumption rates are between 0.13 and 0.30 g g^{-1} h^{-1} for different batch runs. A correlation between the phenol degradation rate and the amount of phenol dose was studied by Vojta et al. (2002). The authors observed that the decrease of the degradation rate was caused by an increase of phenol concentration in addition to other effects. One of them could be the accumulation of intermediates in the reactor (Wang and Loh, 1999).

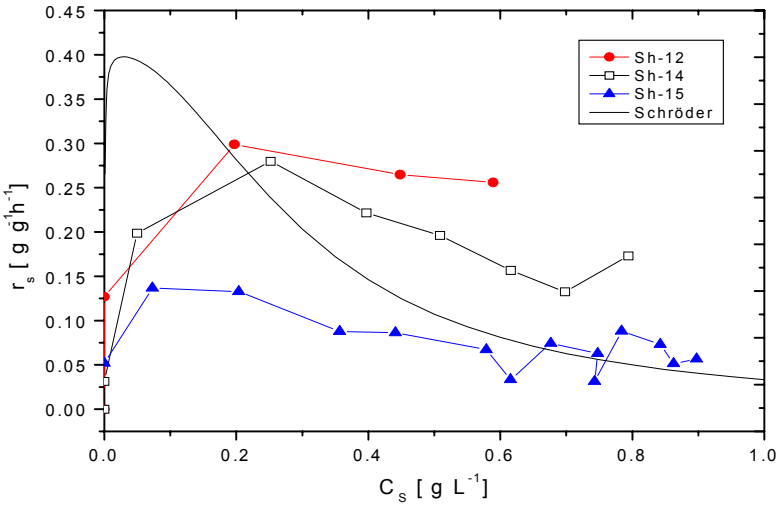

Fig. 4. 16. Specific phenol conversion rates for batch cultivations via phenol concentration. The straight line represents the Yano and Koga kinetics applied by Schröder et al. (1997).

The question remains, whether the intermediate is a dead end product or is, at least partly, converted to biomass and CO_2. With exception of the biomass formation in run Sh-10, considerable biomass formation could not be observed after complete conversion of the substrate. It should be pointed out that carbon recoveries for most of the phenol batch runs increased slightly immediately after depletion of the substrate and remained constant after that. There is also a slightly slower decrease of the CTR compared with OTR after total substrate consumption. This is most probably the reason for the increase of the carbon recovery. The time courses of OTR and CTR reveal, if not caused by a time delay of the CO_2 analyser, that, after depletion of substrate, a small amount of the missing carbon is converted to CO_2 and biomass. Villarejo et al. (1978) found that cyclic Adenosin Mono phosphate (cAMP) in chemostat-grown bacteria is fivefold greater than in batch-grown bacteria. According to its level, cAMP which is controlled by the phosphotransferase system, plays an important role in the mechanism of enzyme regulation in bacterial cells (Schlegel, 1992). Therefore, conversion of the metabolic intermediates was probably reduced or prevented under batch conditions.

4. 2. 1. 1. 2. Wash-out cultivation

To estimate the maximum specific growth rate and the maximum specific substrate consumption rate for phenol, a wash-out experiment was carried out. In a wash-out experiment, the dilution rate of a continuous culture, running in steady state, is increased to a value above the critical one, which equals the maximum attainable growth rate. As a result, the cells are washed out. The maximum rates can than be calculated from the time courses of substrate and biomass. In case of inhibition, the maximum rates are only obtained immediately after the dilution rate switch.

The wash-out experiment was started out of a steady state with a dilution rate of 0.21 h^{-1} and a phenol feed concentration of 2.32 g L^{-1}. The dilution rate was increased to 0.50 h^{-1}, the feed concentration was 2.22 g L^{-1} after the dilution rate step. Fig. (4. 17) shows the biomass, phenol concentration, the CTR and the OTR as function of time after the D-step (t = 0). Immediately after the D-step, the OTR and CTR increase to higher values and remain nearly constant for more than 1.5 h, demonstrating an increased and constant metabolic activity for this time span. During this period, no phenol accumulation occurs, but the biomass concentration is decreasing. After about of 1.8 h, there is a sharp decrease of the OTR and CTR observed, accompanied by an accumulation of phenol and a more rapid decrease of biomass concentration. With exception of the decreasing biomass, there seems to be quasi stationary conditions in the first part of the experiment. The initial biomass concentration is 1.78 g L^{-1}. This corresponds well with the theoretical value of 1.76 g L^{-1} calculated from yield and maintenance coefficients from steady states. Because no inhibition takes place during this time period, the maximum specific growth rate was calculated to 0.47 h^{-1}, and the maximum phenol consumption rate to 0.62 g g^{-1} h^{-1}.

Both values are considerable higher than those determined by Schröder et al. (1997) from unstable steady states (μ_{max} = 0.30 h^{-1}), but comparable with the data of Saéz and Rittmann (1993) with μ_{max} = 0.44 h^{-1} from batch using the same strain on phenol. On the other hand, Léonard et al. (1999) estimated μ_{max} as 0.41 h^{-1} from fed-batch using *Ralstonia eutropha*.

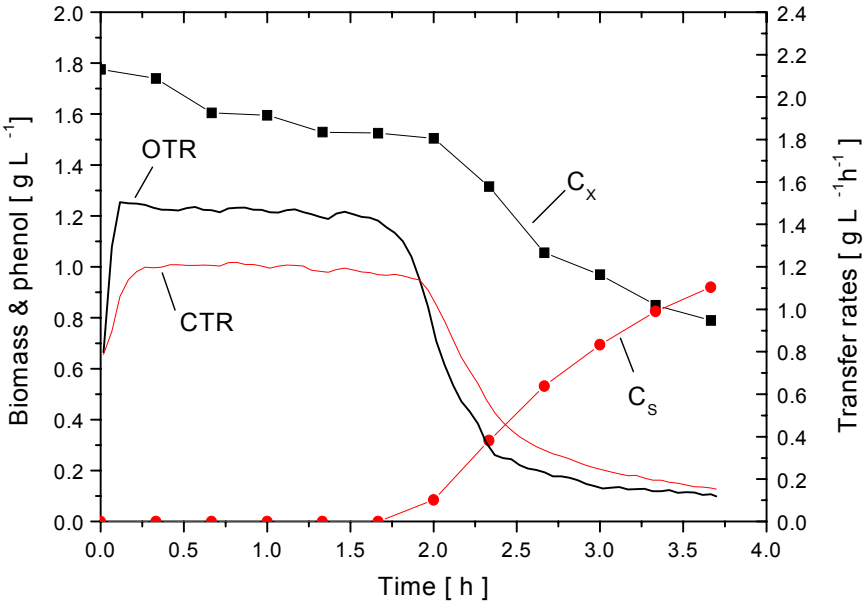

Fig. 4. 17. Experimental data of phenol wash-out at D = 0.50 h^{-1} as functions of time.

With the onset of phenol accumulation at approximately 1.8 h, metabolic activity drops abrutly as is seen in the time courses of OTR and CTR. During this period, increasing phenol accumulation and decreasing specific rates were obtained as a result of an increasing inhibitory effect (Fig. 4. 18). However, the specific rates start to increase again, which can not be explained by a simple substrate inhibition kinetics. This behaviour indicates an adaptation of the cells to higher phenol concentrations, whose effect is contradictory to the increasing substrate inhibition. The time constant of this adaptation indicates a genetic effect. It is most probable that the cells increase their enzyme level. In general, it seems that the culture can grow at a dilution rate of 0.50 h^{-1} for some time. During this time the biomass and the transfer rates decrease slightly, whereas phenol does not accumulate. Eventually inhibition (probably not by phenol) or other metabolic changes take over and act like a switch, reducing metabolic activity considerably. The decrease of biomass in combination with no phenol accumulation in the first 1.8 h reveals that the change in anabolic metabolism is slower than in the catabolic metabolism, which adds another time constant to this already comples system. Or, perhaps, the maximum flow through catabolism and anabolism is different, and phenol starts to accumulate when the maximum specific substrate conversion rate is reached due to the decreasing biomass.

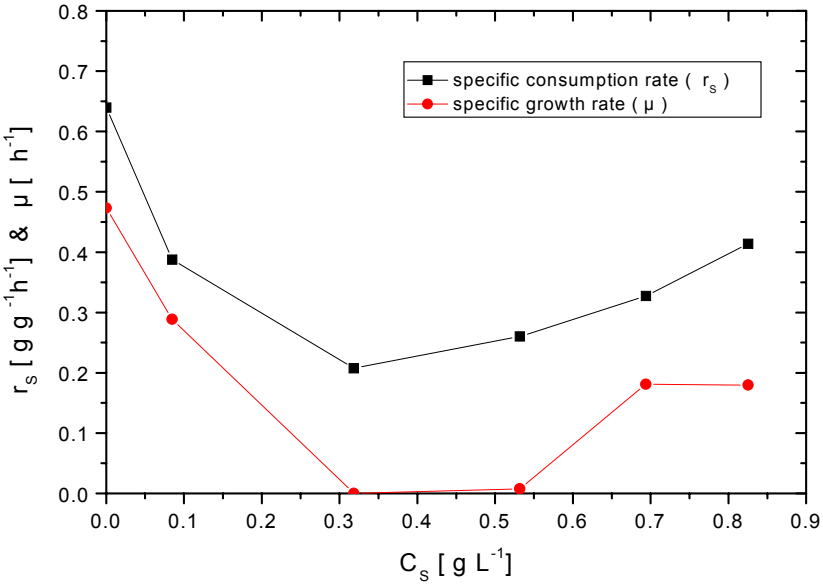

Fig. 4. 18. Specific phenol consumption rate and specific growth rate as functions of phenol concentrations in the wash-out experiment.

The carbon recovery (not shown) decreases to about 0.82 during the first part of the wash-out experiment, and seems to be constant at 0.86 during the second (accumulation) part.

4. 2. 1. 1. 3. A-stat cultivation

To further investigate the phenol wash-out behaviour, an A-stat cultivation was carried out. Starting from a steady state at D = 0.21 h^{-1} with a phenol feed concentration of 2.32 g L^{-1}, the dilution rate was increased linearly over 6.7 h using an acceleration factor of a = 0.05 h^{-2}. During the duration of the experiment, the phenol feed concentration dropped slightly to 2.30 g L^{-1}. The experimental data of the A-stat cultivation are given in Fig. (4. 19). No- phenol accumulation was observed in the first 5.5 h, and the OTR and CTR increased linearly. The onset of accumulation corresponded with a dilution rate of 0.48 h^{-1}. So, the maximum attainable growth rate in this experiment is comparable to the value obtained from the wash-out experiment.

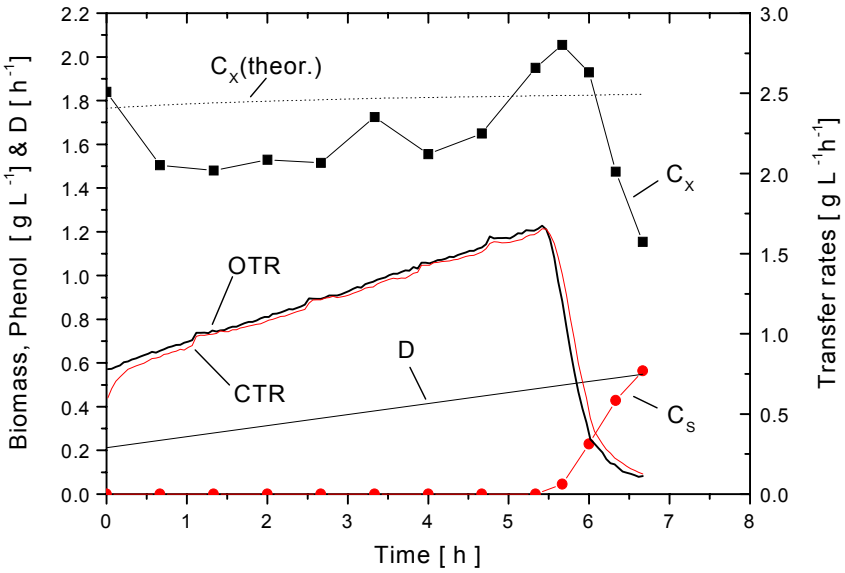

Fig. 4. 19. Experimental data for phenol ramp (A-stat) cultivation as functions of time.

The carbon recovery was not calculated for this experiment because of the high data scatter of the biomass values. The biomass seems to drop from 1.84 g L^{-1} to 1.51 g L^{-1} after initiating the A-stat. After that, the biomass increases slightly, but shows a prominent increase at approximately 6.0 h, shortly before accumulation of phenol. The dashed line in Fig. (4. 19) represents the theoretical biomass calculated from the yield and maintenance values derived from the chemostat. The experimental values are located above and below this line, therefore it can be assumed that the cells are growing with their maximum yield as in a true chemostat, and that the carbon recovery is fulfilled. The validity of this assumption was proved later with the binary and ternary substrate mixtures (see chapters 4.2. 2. 2. 2 and 4. 2. 3. 2).

The specific growth and substrate consumption rates are plotted in Fig. (4. 20). The values of the growth rate are influenced by the biomass data scatter, but seem to correspond well with the dilution rate. The course of the specific phenol consumption rate also follows a straight line which is roughly parallel to the dilution rate. The small deviation is caused by the slightly increasing experimental yield due to decreasing influence of maintenance with increasing growth rate.

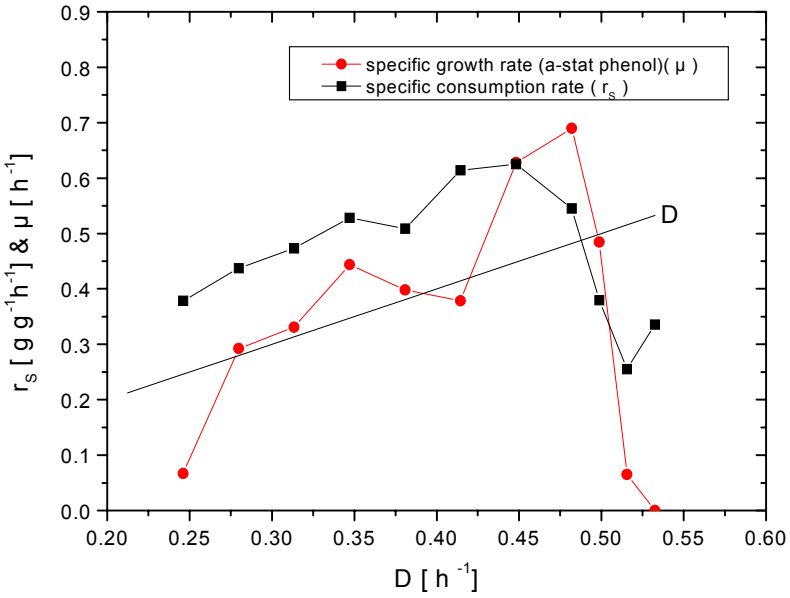

Fig. 4. 20. Calculated specific growth rate and specific phenol consumption rate versus the dilution rate during the phenol A-stat cultivation.

Paalme et al. (1995) investigated A-stat cultivations at various acceleration factors. The authors stated that an A-stat with an acceleration factor between 0.01 to 0.05 h^{-2} is too fast to provide steady state conditions. Whether or not the growth conditions in this work resemble steady state conditions, is not yet quite clear.

The maximum specific growth rate reached approximately 0.50 h^{-1} during wash-out and A-stat conditions, considerably higher than the value obtained by Schröder et al. (1997). Reuss and Fisher (1991), also obtained higher growth rates using *Pseudomonas putida*. It seems that the cells are able to grow at higher rates under certain conditions. Growth rates in the vicinity of 0.50 h^{-1} were not observed in batch cultivations, where substrate inhibition reduces metabolic rates. But it is not only substrate inhibition, because at low substrate concentrations (end of batch), the growth rate was also considerably lower than 0.50 h^{-1}. The higher growth rates were obtained at conditions where the substrate concentration was below detection limit. From the results of the wash-out, it seems also that the cells are capable to adapt to changing conditions, but with different time constants for the catabolic and anabolic routes.

The regulatory factor may therefore be either the substrate concentration, which allows higher rates only at very low substrate concentration to prevent overload of the slower adapting anabolic routes, or a pathway product for the same reason. Perhaps the slower adapting anabolic pathways lead to the accumulation of regulatory compounds during the wash-out experiment, which after reaching a certain level, reduced metabolic activity.

The maximum growth rate of 0.30 h^{-1} obtained by Schröder et al. (1997) seems to be a constant value, which can be achieved under different conditions and for long times.

The carbon recovery is fulfilled at steady state (Schröder et al. 1997, and this work) and at A-stat (this work) conditions, but deviations were observed at wash-out and batch conditions. It seems therefore that formation of unknown metabolites is the result of sudden changes of culture conditions, most probably due to the different time constants of catabolism and anabolism. But the accumulating compound or compounds is not the reason for the growth rate regulation, because the carbon recovery is decreasing during the first 1.5 h of the wash-out cultivation, whereas the culture is growing constantly at 0.47 h^{-1} during this time span.

In general, it can be stated, that conversion of phenol by *B. cepacia* G4 is a complex process which can not be described by simple kinetics. It seems evident that the performance of the cells is dependent of the culture history, substrate inhibition, adaptation (time dependent effects), and probably metabolic regulation. Additionally, intermediate formation plays an important role for phenol degradation.

4. 2. 1. 2. Assessment of the time delay for the exhaust gas in the reactor

As a general result, the carbon recovery, which is fulfilled at steady states, shows a depletion during the dynamic experiments. In order to investigate, whether this depletion was caused by metabolic functions (intermediate formation) or by physical effects, the exhaust gas analysis was checked for time delay effects during batch Sh-12 for instance (Fig. 4. 21). The exhaust gas, leaving the culture suspension, passed the following delaying stages before entering the analyser:

1) reactor head room, V = 1.2 L
2) exhaust gas cooler, V = 0.5 L
3) tubing, 100cm x 8mm
4) foam trap, V = 5.0 L
5) tubing, 80cm x 8mm
6) exhaust gas filter, V = 1.0 L
7) tubing, 400 cm x 8mm
8) tubing, 900 cm x 6mm

Plug flow was considered for all tubings, and well mixing (Pt1-delay) for all other stages. The combined delay for all plug flow stages (tubings) was calculated to approximately 12 s, which can be neglected for the purpose of this work. For the well mixed stages, the following equations can be applied:

$$dy_{(t)}/dt = F/V\ (x_{(t)} - y_{(t)}) \qquad [4.\ 8]$$

with $x_{(t)}$ being the input variable and $y_{(t)}$ being the output variable. Solved for $x_{(t)}$:

$$x_{(t)} = y_{(t)} + V/F\ (dy_{(t)}/dt) \qquad [4.\ 9]$$

The CTR before entering a delaying stage can be calculated for each single delaying stage from the CO_2-analyser back to the reactor. For the CO_2-analyser, a Pt1-behaviour was assumed (time constant = 5 s, Kies, 1998). Fig. (4.21a) shows a comparison between the experimental CTR calculated from the analyser data and the corrected CTR without any delay stages for batch run Sh-12. The carbon recovery for Sh-12 was recalculated using the corrected CTR data (Fig. 4.21b). Both curves are nearly identical. The effect of all delaying states in the exhaust gas measurement can therefore be neglected. This means, that the deviation of the carbon recovery in the dynamic experiments is not caused by time delays but by formation of intermediate(s).

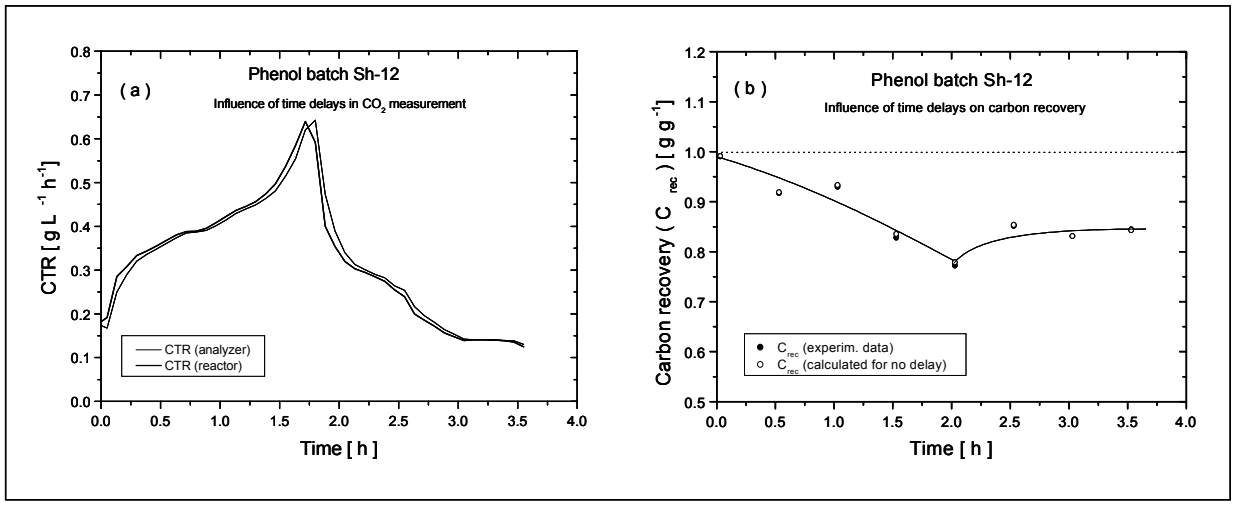

Fig. 4. 21. Influence of the time delays in measurement of both carbon transfer rate (a) and carbon recovery (b) calculated for phenol batch Sh-12 cultivation.

4. 2. 1. 3 Biological degradation of benzoate

4. 2. 1. 3. 1. Batch cultivation

Benzoate batch experiments were carried out as described in the previous chapter for phenol. Table (4. 5) shows the data for the three experiments. The runs were carried out using different initial benzoate concentrations and at different dilution rates of the preceding chemostat. For the batches Sh-17 and Sh-19, the benzoate feed concentration in the chemostat, which determines the initial biomass concentration, was the same, but for Sh-20 it was higher. During all benzoate runs, a yellow colour showed up in the bioreactor, indicating formation of 2-hydroxymuconic semialdehyde, the reaction product of catechol by the *meta* pathway enzyme 2,3-dioxygenase. The same phenomenon was also observed during cultivation of benzoate under steady state conditions.

Table 4. 5. Culture history and initial benzoate concentrations used in the batch experiments.

Experiments	Culture history			C_{S0}-batch g L^{-1}
	D-chem. h^{-1}	C_{S0}-chem. g L^{-1}	C_X-chem. g L^{-1}	
Sh-17	0.052	1.873	1.223	0.6731
Sh-19	0.104	1.873	1.320	0.9871
Sh-20	0.223	2.017	1.457	1.1874

For the first batch (Sh-17), a benzoate concentration of 0.67 g L^{-1} was used (Fig. 4. 22). The substrate was completely converted after 1.7 h, resulting in a biomass increase from 1.3 to 1.6 g L^{-1}. The time courses of OTR and CTR are more flat than for phenol, indicating only minor influence of inhibition. Carbon recovery data (not shown) show a depletion as was detected for phenol. The minimum (C_{rec} = 0.89) was also observed when the substrate was completely used up. The C_{rec} did not increase again, indicating formation of a dead end metabolite. This is also supported by the biomass, which did not increase after complete conversion of the substrate. In context with the C_{rec} data, the biomass yield is lower compared to steady state data.

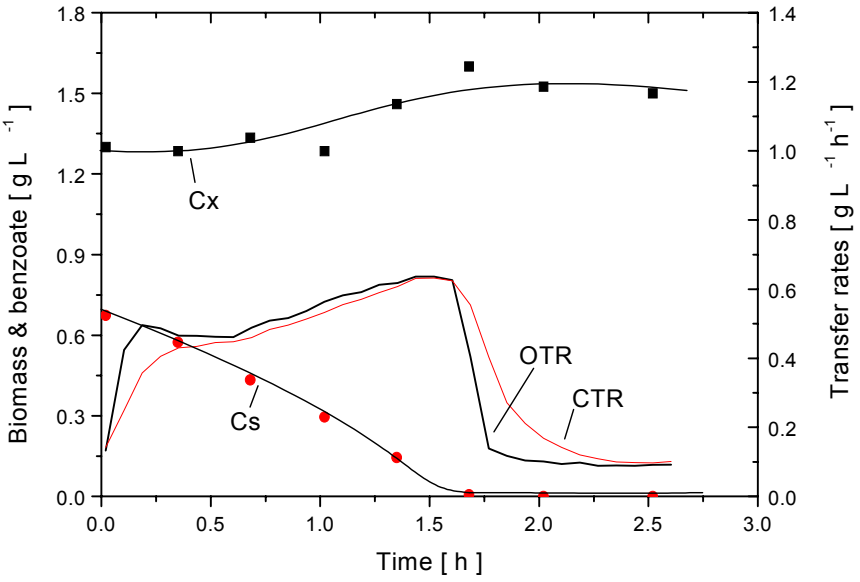

Fig. 4. 22. Experimental data obtained from batch cultivation Sh-17 on benzoate.

The second run (Sh-19) is shown in Fig. (4. 23). The initial benzoate concentration was 0.99 g L^{-1}. The general outline is similar to that of Sh-17, but the time courses of OTR and CTR show a prominent peak at the beginning of the runs. Whether this is just some kind of oscillation or the onset of inhibition, has to be investigated. The carbon recovery data are plotted in Fig. (4. 24). Here too, the data indicate formation of dead end metabolites. This is in conjunction with the biomass data and the sharp decrease of OTR and CTR after 2.5 h. The minimum of the C_{rec} is about 0.82.

The third run (Sh-20) is shown in Fig. (4. 25). Here, the initial biomass is higher because a higher benzoate feed concentration in the preceding chemostat was supplied. 1.19 g L^{-1} benzoate are consumed in 2 h, which is faster than in the previous batch. Whether this is caused by the higher initial biomass or by the higher chemostat dilution rate will be investigated in chapter 4. 2. 1. 3. 3 (Modelling of benzoate cultivation). The peak in the initial phase of the time courses of CTR and OTR is not so prominent here as in Sh-19. The carbon recovery (not shown) decreased and reached its minimum of 0.79 at the same time as benzoate is completely used up, and remains constant for the rest of the cultivation. This run also strongly supports formation of dead end metabolites.

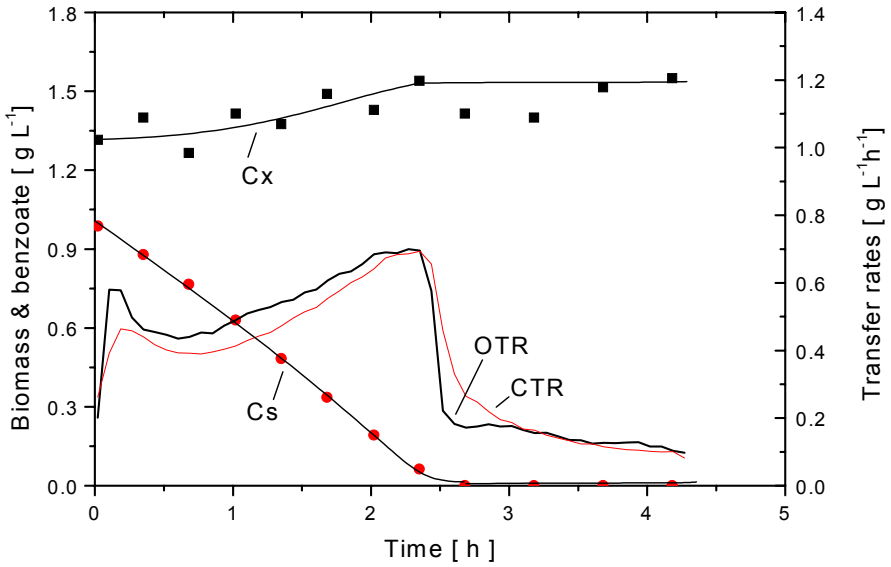

Fig. 4. 23. Experimental data obtained from batch cultivation Sh-19 on benzoate.

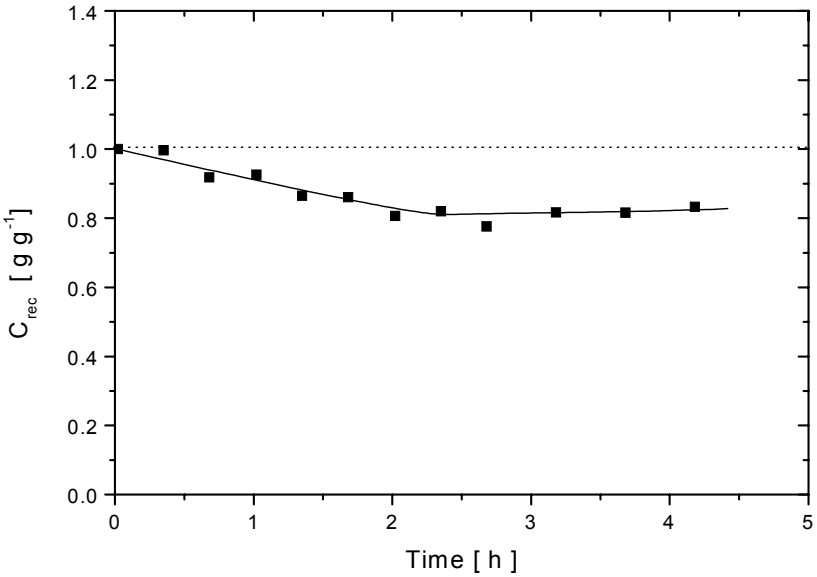

Fig. 4. 24. Carbon recovery for batch cultivation Sh-19 on benzoate.

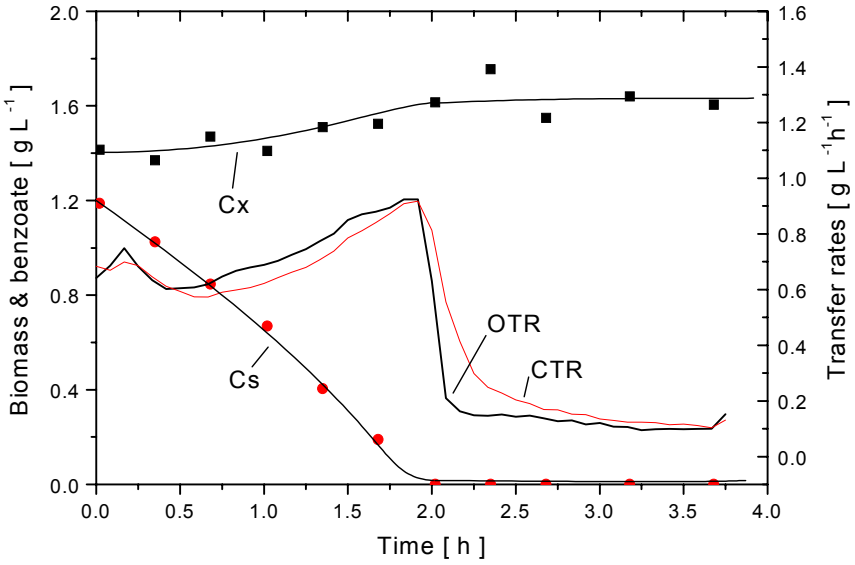

Fig. 4. 25. Experimental data obtained from batch cultivation Sh-20 on benzoate.

Fig. (4. 26) shows a comparison of the three batches. As for phenol, the batch performed out of the chemostat with the highest dilution rate shows a deviation to the other runs.

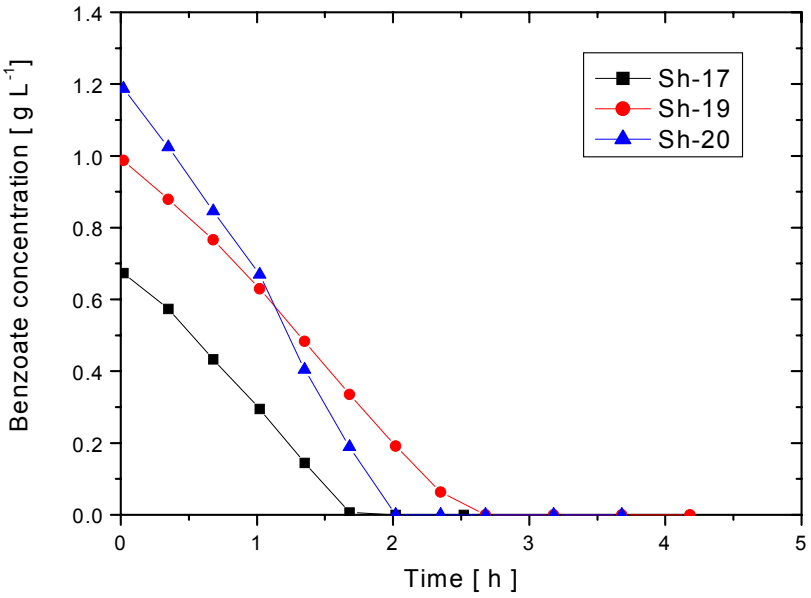

Fig. 4. 26. Comparison of the time courses for substrate conversion of the benzoate batch cultivations.

However, the direction of this deviation is contrary compared with the phenol cultivations. For phenol, run Sh-15, originating from a higher dilution rate of the preceding chemostat than Sh-14, shows a slower conversion. For benzoate, conversion of Sh-20, also originating from a higher dilution rate than Sh-19, is considerably faster. So, the results of the benzoate experiments are more in conjunction with the theory of Kovárová-Kovar and Egli (1998), who assume that cell performance is influenced by adaption and "training" of the cells. This is also demonstrated in Fig. (4. 27), which shows the specific benzoate conversion rate as a function of the benzoate concentration for the three experiments. The specific rate of Sh-20 reaches higher values than those of the other batches, showing the effect of a better training / adaptation (higher growth rate of the preceding chemostat).

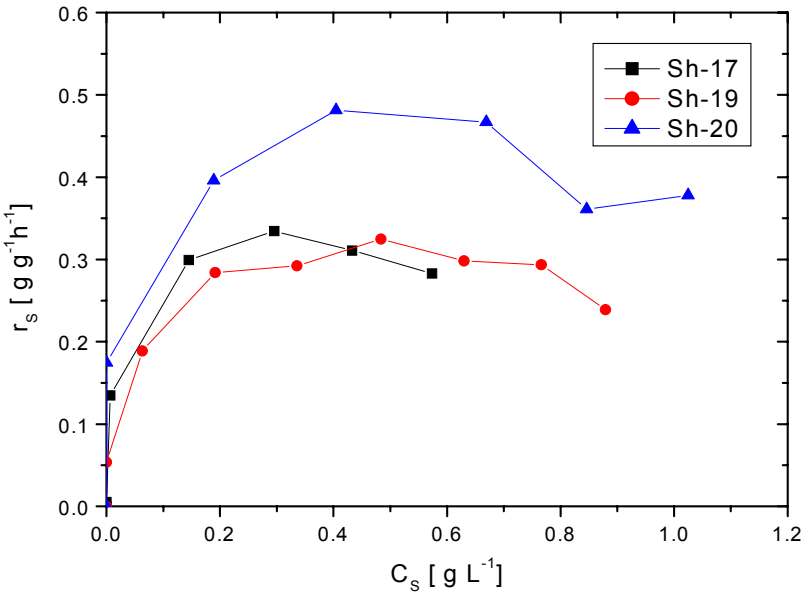

Fig. 4. 27. Specific benzoate consumption rates for batch cultivations via benzoate concentration.

The achieved maximum values of the specific consumption rate are 0.30 g g^{-1} h^{-1} for Sh-17 and Sh-19, and 0.48 g g^{-1} h^{-1} for Sh-20. These values are in good agreement with the data obtained by Ampe and Lindly (1995 & 1996) for *Alcaligenes eutrophus*. Here, it is interesting to mention that the use of a higher initial concentration of benzoate leads to a higher maximum specific degradation rate. This is in accordance with the dependency of r_S on the benzoate concentration (Fig. 4. 27). It indicates that, in contrast to phenol, there is no or only a slight substrate inhibition in the investigated concentration range of benzoate. The carbon recoveries show, as was already observed for phenol, a deviation with a minimum at the time of total substrate conversion. The carbon recoveries are constant after that, so the formed metabolites are not further converted to biomass and carbon dioxide.

4. 2. 1. 3. 2. Wash-out cultivation

Using benzoate as a sole source of carbon, a wash-out experiment was carried out. The experimental results are shown in Fig. (4. 28). The preceding steady state was performed at D = 0.26 h^{-1} using a benzoate feed concentration of 1.92 g L^{-1}. At t = 0, the dilution rate was increased to D = 0.42 h^{-1}. The benzoate feed concentration was 1.97 g L^{-1} during wash-out conditions.

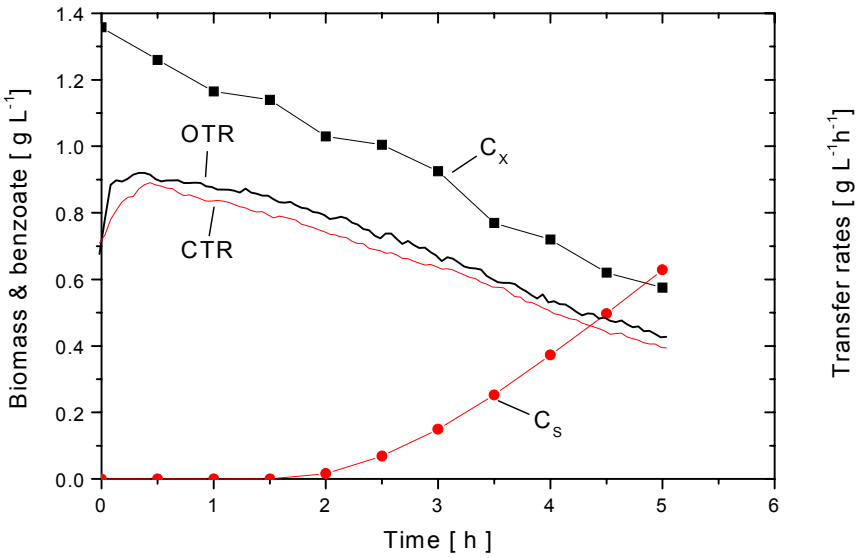

Fig. 4. 28. Experimental data of benzoate wash-out at D = 0.42 h^{-1}.

After the step increase of the dilution rate, the biomass concentration decreased steadily. The same applied for the OTR and the CTR, after their brief increase as a result of the higher substrate feed. Benzoate accumulation started after about 1.5 h. In contrast to the phenol wash-out, a dilution rate of 0.42 h^{-1} was higher than the critical dilution rate. The delayed appearance of benzoate supports the observation from the wash-out on phenol, that the time constants for adaptation are different for growth and substrate conversion, or that the maximum flows through catabolism and anabolism are different. In the batch experiments, no substrate inhibition of benzoate was observed in the investigated concentration range. Fig. (4. 29) shows the calculated specific rates as a function of the benzoate concentration. There is only a slight decrease of the specific rates, so there may be a slight benzoate substrate inhibition, but it is not very significant. The maximum specific growth rate (μ_{max}) at the beginning of the wash-out is 0.30 h^{-1}, which corresponds well with the data given by Schröder et al. (1997) for phenol. The specific benzoate conversion rates are approximately 0.70 g g^{-1} h^{-1} during the whole experiment, which results in a biomass yield of only 0.43 g g^{-1}. This is considerably lower than expected from the steady state results. Ampe and Lindley (1996) investgated benzoate conversion by *Alcaligenes eutrophus* and did not observe any substrate inhibition.

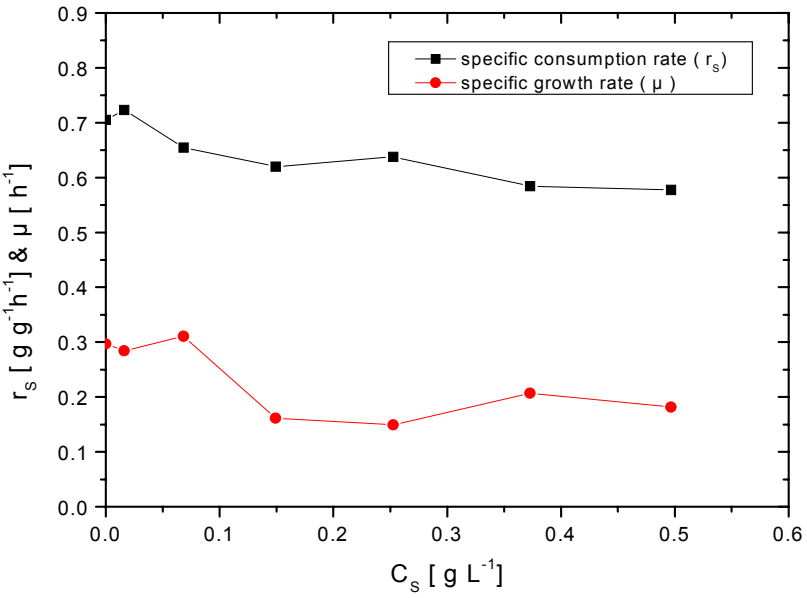

Fig. 4. 29. Specific rates of growth and benzoate consumption during wash-out cultivation with benzoate as a sole source of carbon.

The carbon recoveries decrease to 0.78 in the first 2.0 h (Fig. 4. 30), but remain constant at this value during accumulation of benzoate, indicating accumulation of metabolites. The same outcome was also achieved during all batch experiments, whereas at steady state the C_{rec} is fulfilled.

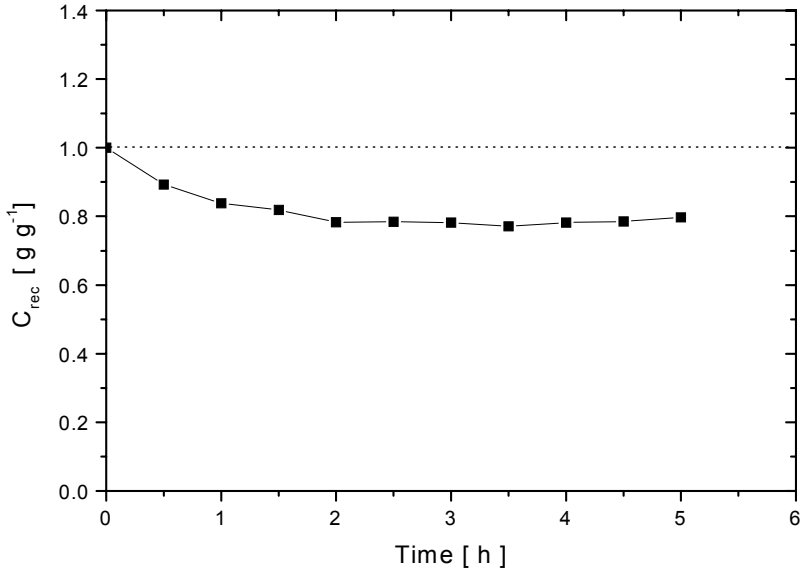

Fig. 4. 30. Carbon recovery recorded during benzoate wash-out cultivation at D = 0.42 h^{-1}.

Based on the results obtained, benzoate conversion to biomass and carbon dioxide was complete at steady states. Under excess of substrate conditions (batch) and high growth rates (wash-out, initial phase), formation of metabolites was observed. It is not yet clear if this is solely caused by an overflow (higher substrate conversion than corresponding growth), or by a change of metabolic routes or regulation. Phenol and benzoate are assumed to be metabolized by the same degradation sequence (pathway), resulting in the same metabolites of the intermediate metabolic pathway (pyruvate and acetaldehyde). Whereas the growth on benzoate seems to be limited at 0.30 h^{-1}, the cells are able to grow on phenol at specific growth rates of up to 0.50 h^{-1}, at least for some time. At unstable steady states, however, growth is also limited to 0.30 h^{-1} (Schröder et al. 1997). It seems that 0.30 h^{-1} is a stable growth rate which can be performed by the cells for a long time, whereas, starting from a steady state, the cells are able to metabolize phenol for a short time at higher rates.

Although phenol and benzoate are mineralized via the same metabolic pathway (*meta*), a yellow colouration was observed in the medium during cultivation on benzoate, but not on phenol. The reason for the difference is not yet known. Perhaps, different enzymes (parallel routes) were induced in the *meta*-pathway during phenol cultivation, or the conversion step of catechol to 2-hydroxymuconic semialdehyde was repressed or inhibited by phenol, therefore the yellow dye was not observed.

4. 2. 1. 3. 3. Modelling of benzoate cultivation

The aim of this chapter is to establish a simple mechanistic model for benzoate degradation which includes adaptation of metabolic activity of a continuous culture of *B. cepacia* G4 subjected to a sudden increase of both the dilution rate (wash-out) and the benzoate concentration (batch), in especially to prove if the theory of Kovárová-Kovar and Egli (1998) is valid for this biological system.

Compared with phenol, degradation of benzoate seems somewhat simpler. Growth rates above 0.30 h^{-1} were not observed and the influence of the culture history seems to follow the theory of Kovárová-Kovar and Egli (1998), leading to a higher performance in the batch cultures if the cells were better adapted to high growth rate (higher dilution rate in preceding chemostat). Therefore it was assumed that the influence of the culture history can be expressed as a function of the growth rate, which equals the dilution rate of the chemostat.

For simplicity, the unknown metabolites were expressed as a single metabolite. The carbon recovery data were used to calculate the time dependent concentration of these "intermediates". Because of the unknown stoichiometry and molecular weight of the intermediate, the intermediate concentration was expressed as the mass of carbon in the intermediates per litre:

$$C_i = a_i \; C_{int} \qquad [4.\ 10]$$

with a_i = mass of carbon / mass of intermediate(s) [4. 11]

and C_{int} = concentration of intermediate(s) in g L^{-1} [4. 12]

For modelling, simulation, and data fitting, the MATLAB software platform together with the BIOSS-(Biotechnological Simulation System)-toolbox was used. The model consists of the following equations:

Mass balances:

$$dC_S / dt = D\,(C_{S\,0} - C_S) - r_S\,C_X \qquad \text{substrate} \qquad [4.\ 13]$$

$$dC_X / dt = (\mu - D)\,C_X \qquad \text{biomass} \qquad [4.\ 14]$$

$$dC_i / dt = r_i\,C_X - D\,C_i \qquad \text{intermediate(s)} \qquad [4.\ 15]$$

Kinetics:

$$r_S = r_{S\,max}\,C_S / (K_S + C_S + C_S^2/K_i) \qquad \text{substrate} \qquad [4.\ 16]$$

$$\mu = f\,Y_{X/S}\,r_S - Y_{X/S}\,m_S \qquad \text{biomass} \qquad [4.\ 17]$$

$$r_i = Y_{i/S}\;r_S \qquad \text{intermediate(s)} \qquad [4.\ 18]$$

$$r_{CO2} = Y_{CO2/S}\,r_S \qquad \text{carbon dioxide} \qquad [4.\ 19]$$

The CTR was simulated by a linear equation:

$$CTR = r_{CO2}\;C_X \qquad [4.\ 20]$$

and the assumption, that the carbon dioxide transfer rate equals the carbon dioxide uptake rate.

A substrate inhibition kinetics (Andrews, 1968) was used, because the increasing time courses of OTR and CTR can not be modelled with a Michaelis-Menten equation. For growth kinetics, the Pirt equation (Pirt, 1965) was used. This equation was derived to describe substrate limited growth in a chemostat. To incorporate reduced growth in batch and formation of intermediate, the growth kinetic equation was modified by adding the factor f, which describes deviation from chemostat conditions. Formation of intermediate and carbon dioxide were considered as substrate conversion related. Factor f in the growth kinetics assumes that a fixed amount of substrate is routed into the intermediate(s). This assumption has to be proved yet, therefore the modelling was carried out stepwise.

In the first step, only benzoate conversion was modelled by fitting the model equation to the experimental data. Only the substrate mass balance was integrated numerically. For the biomass concentration the experimental data or interpolated values of the experimental data were used, respectively. Each experiment, the three batches and the wash-out were fitted individually. The results are shown in Table (4. 6). The lower bound of the fitting algorithm was set to 10^{-5}, the estimated K_S-values are equal or lower than this value. It is clear that non of the experiments is sensitive to a variation of K_S, therefore K_S was fixed to 10^{-5} g L^{-1}. A parameter sensitivity analysis (not shown) also revealed a linear dependency between r_{Smax} and K_i, which means that both parameters can not be estimated independently. Therefore, K_i was also fixed to 1.5 g L^{-1} for all experiments and the data fitting repeated. The resulting $r_{S\,max}$-values are given in the last column of Table (4. 6).

Table 4. 6. Kinetic parameters of the individual simulation for different cultivations on benzoate.

Experiments	D-chem. h^{-1}	Individual simulation with:		
		fixed K_S		fixed K_S & K_i
		$r_{S\,max}$ g $g^{-1}h^{-1}$	K_i g L^{-1}	$r_{S\,max}$ g $g^{-1}h^{-1}$
Sh-17	0.052	0.441	0.845	0.377
Sh-19	0.104	0.482	0.892	0.390
Sh-20	0.223	0.617	1.398	0.599
Wash-out	0.257	0.706	1.612	0.705

According to the theory of Kovárová-Kovar and Egli, cells can adapt to substrate limited and substrate surplus growth by varying their kinetic and stoichiometric parameters. The variation can include all parameters: $r_{S\,max}$, K_i and K_S. Because $r_{S\,max}$ and K_i can not be estimated separately, the question was wether to fix K_i or $r_{S\,max}$.

A variation of $r_{S\,max}$ can be considered as a change of the enzyme concentration in the cell by increased or reduced transcription / translation. This genetic regulation has time constants in the range of minutes to hours, which is in accordance with the experimental observation. Enzymatic regulation, on the other hand, has time constants in the range of milliseconds. The variation of $r_{S\,max}$ may therefore be expressed as:

$$r_{S\,max} = K_{cat}\ E \qquad [4.\ 21]$$

with K_{cat} being the velocity constant and E being the enzyme concentration in the cell. So it was decided to fix K_i and estimate $r_{S\,max}$.

$r_{S\,max}$ increases with increasing specific growth rate (dilution rate) of the preceding chemostat. Because of the unknown biological relationship between $r_{S\,max}$ and D-chem, a simple power function was used to describe the variation of $r_{S\,max}$ as a function of D-chem:

$$r_{Smax} = P1 + P2\ \text{D-chem} + P3\ \text{D-chem}^2 \qquad [4.\ 22]$$

The three parameters were obtained by non linear data fitting:

P1 = 0.41201 ± 0.00055
P2 = -1.1319 ± 0.00924
P3 = 8.83879 ± 0.02942
Chi^2 = 4.2878E-8
R^2 = 1

In the second step, the yield coefficients $Y_{CO2/S}$ and $Y_{i/S}$ were estimated also using the experimental values for the biomass. The results are given in Table (4. 7).

Table 4. 7. Individual and common fitting of yield coefficients ($Y_{CO2/S}$ and $Y_{i/S}$) as well as f-factors for different cultivations on benzoate.

Experiments	$Y_{CO2/S}$ g g^{-1}	$Y_{i/S}$ g g^{-1}	f factor
Sh-17	1.027	0.220	0.399
Sh-19	1.114	0.275	0.261
Sh-20	0.891	0.274	0.213
Wash-out	1.078	0.229	0.572
Common fitting	1.072	0.255	0.556

Here fore, the chemostat values of $Y_{X/S}$ and m_S were used, the K_S and K_i were fixed to 10^{-5} and 1.5 g L^{-1} respectively, and $r_{S\,max}$ was calculated by equation [4. 22]. All mass balances without the one for biomass were integrated numerically, and the parameter identification was done for each experiment individually and for the combination of all experiments. The results in Table (4. 7) show only a slight deviation of the parameters $Y_{CO2/S}$ and $Y_{i/S}$ for the individual experiments, indicating that carbon dioxide and intermediate formation can be described by a constant yield coefficient.

In the last step, factor f was estimated by integrating all mass balances and using the parameters estimated so far. The resulting values for f (individual and common fitting) are also given in Table (4. 7). In contrast to the yield coefficients, f shows a considerable deviation for the individual experiments, ranging from 0.21 to 0.57. A comparison of experimental data and model is shown in Fig. (4. 31).

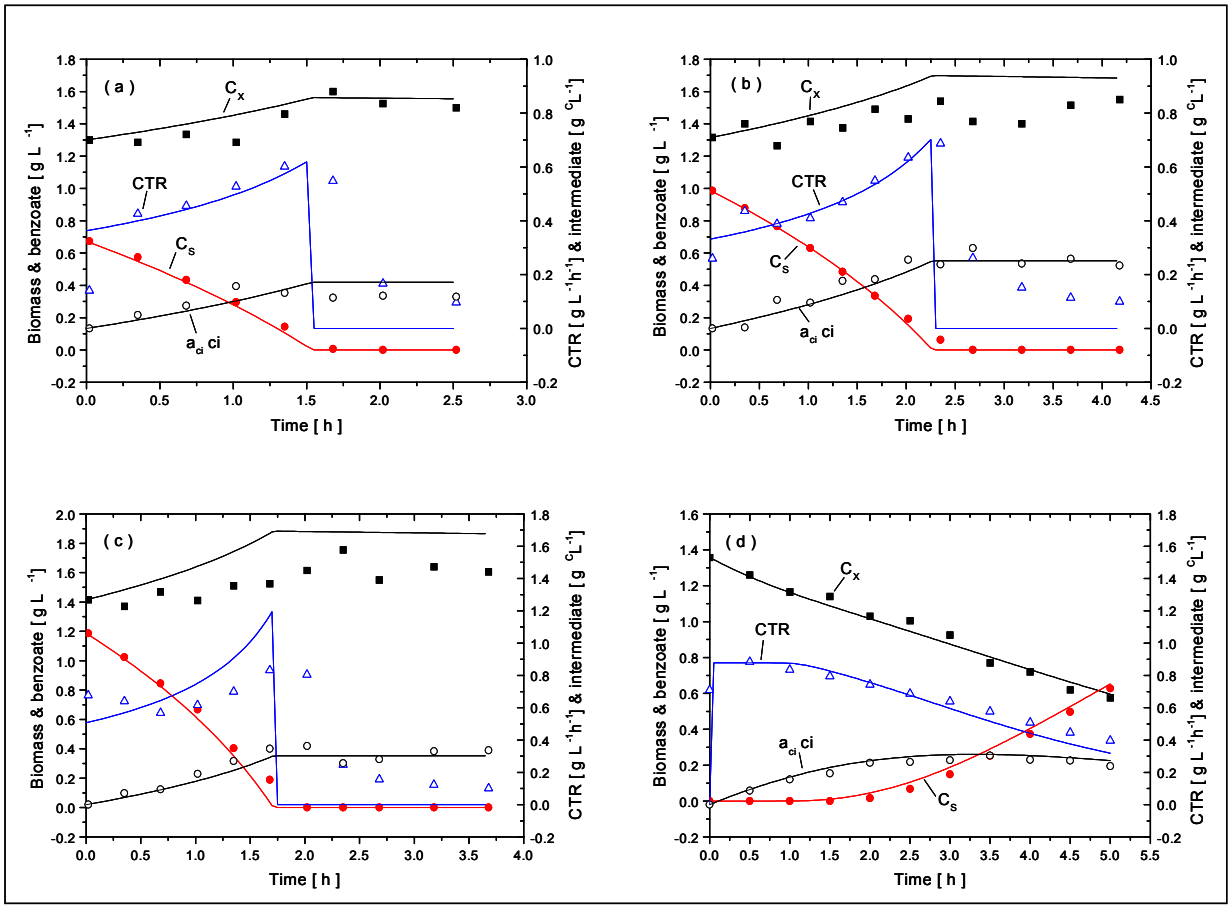

Fig. 4. 31. Experimental data (symbols) and model simulation (lines) of different cultivations on benzoate: batch experiments Sh-17, Sh-19, and Sh-20 (a, b and c, respectively) and the wash-out experiment (d).

For all simulations, the common f factor was used. Good agreement between model and experimental data was achieved for all variables for experiments Sh-17 and the wash-out. For Sh-19, only the biomass shows some variation. For Sh-20, the simulation results for biomass and CTR are slightly above the experimental data, all other variables show good agreement. The deviations of Sh-19 and Sh-20 are caused by the f factor, who shows the highest deviation from the common value in these experiments.

In general, the simple model can describe benzoate conversion under different conditions (batch, wash-out, different culture history) quite well. It also supports the theory of Kovárová-Kovar and Egli (1998), Senn et al. (1994) and Sokoł (1987) showing the importance of the culture history on the performance of the cells. The expected time delay between catabolism and anabolism was not incorporated in the model. However, the time course of the CTR shows some difference between model and experimental data. Whereas the model predicts an abrupt decrease of CTR after complete conversion of substrate, the experimental data decrease more slowly. This indicates that carbon dioxide production is related to growth and that carbon dioxide production continuous for some time after complete depletion of substrate, most probably due to the different time constants.

4. 2. 1. 4. Biological degradation of acetate

4. 2. 1. 4. 1. Batch cultivation

With sodium acetate as sole source of carbon, three batch experiments were carried out. The data are shown in Table (4. 8). Because of the lower yield compared with phenol and benzoate, chemostat feed concentrations of sodium acetate were set at higher values to increase the biomass concentration. All batches were carried out from different dilution rates. Also, the initial concentrations of sodium acetate were higher compared with the other substrates.

Table 4. 8. Culture history and initial sodium acetate concentrations used in the batch experiments.

Experiments	Culture history			C_{S0}-batch g L^{-1}
	D-chem. h^{-1}	C_{S0}-chem. g L^{-1}	C_X-chem. g L^{-1}	
Sh-21	0.048	3.802	1.079	1.063
Sh-23	0.094	3.771	1.249	2.215
Sh-24	0.205	2.950	1.073	3.347

Fig. (4. 32) shows a comparison of the three runs. Here, the batch out of the highest dilution rate fits well to the other experiments. Compared to phenol and benzoate, sodium acetate conversion involves different catabolic enzymes.

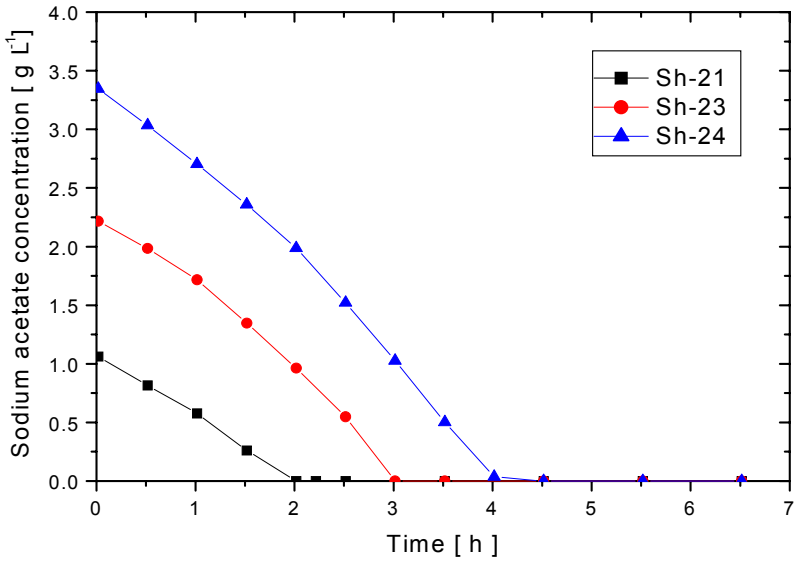

Fig. 4. 32. Time courses of sodium acetate conversion for the three batch experiments.

The first batch (Sh-21) is shown in Fig. (4. 33). Approximately 1.1 g L^{-1} of sodium acetate is consumed in 2 h, resulting in a biomass increase from 1.17 g L^{-1} to 1.28 g L^{-1}. As for the two other substrates, the yield is considerably lower than at steady state. Increase of biomass and of both transfer rates is corresponding, no inhibition towards conversion of sodium acetate was observed. After substrate depletion, OTR and CTR drop sharply, and the biomass seems to be constant for the next hours.

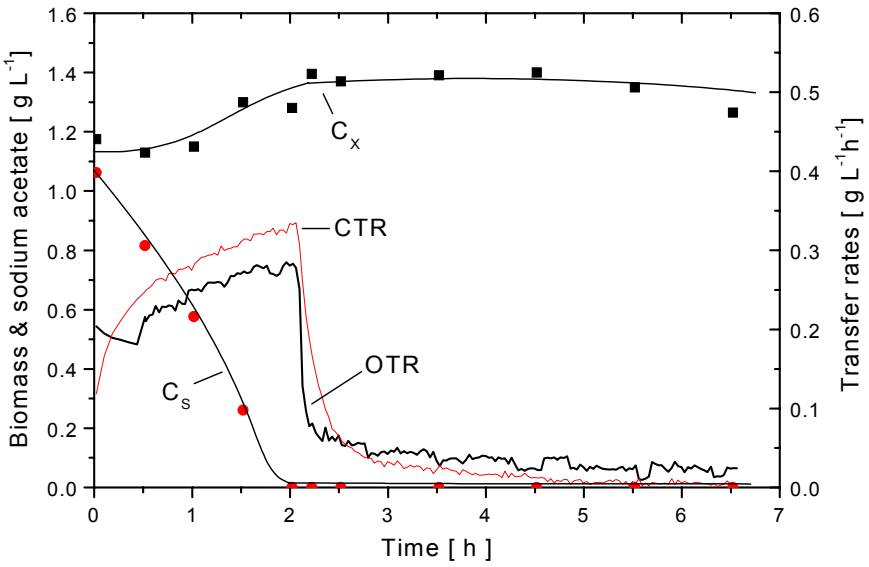

Fig. 4. 33. Experimental data for batch cultivation Sh-21 on sodium acetate.

The carbon recovery decreases to about 0.86 (Fig. 4. 34), but in contrast to phenol and benzoate, increases again after complete substrate conversion. After four hours, the carbon recovery decreases again. This is accompanied by a biomass decrease, therefore the cause for the decrease of C_{rec} after four hours is most probably not a formation of intermediates but turnover of biomass.

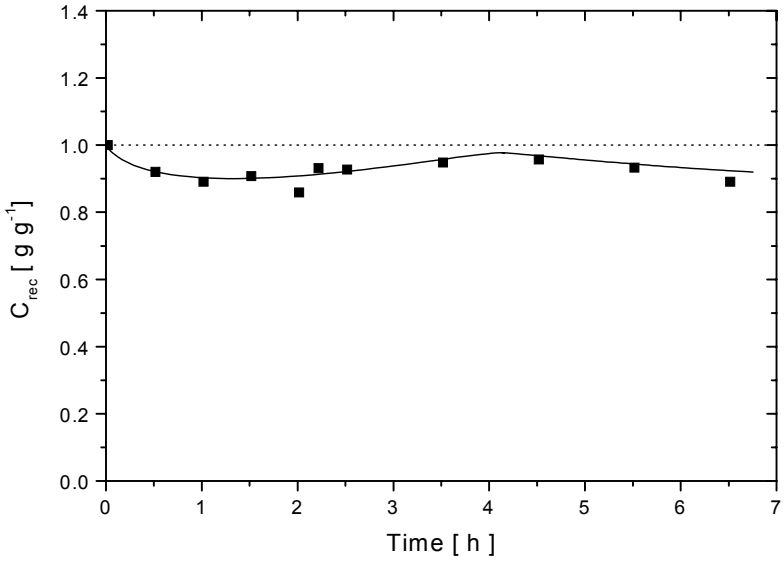

Fig. 4. 34. Carbon recovery data for batch cultivation Sh-21 on sodium acetate.

The second and the third experiments (Sh-23 and Sh-24) are shown in Figures (4. 35) and (4. 36), respectively. Sh-23 was carried out with an initial sodium acetate concentration more than twice as high compared with the first batch.

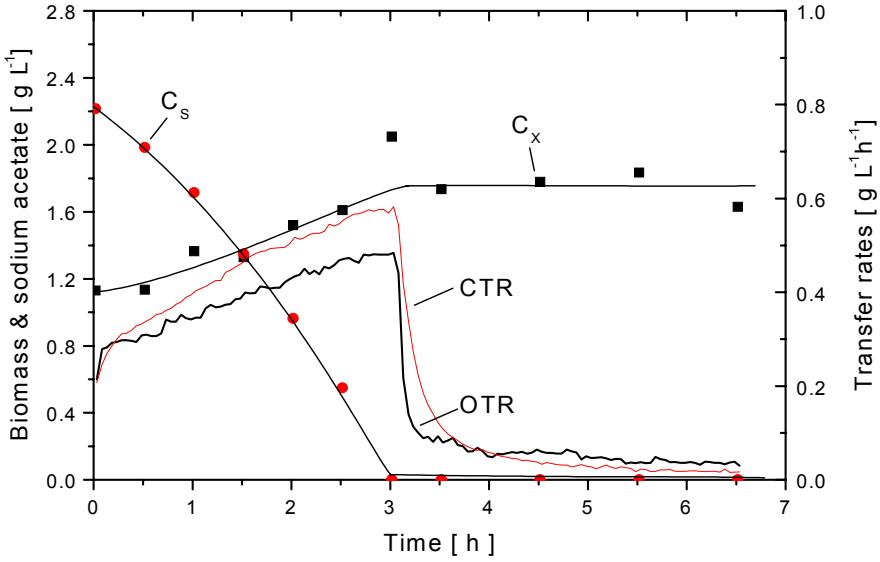

Fig. 4. 35. Experimental data for batch cultivation Sh-23 on sodium acetate.

Biomass, CTR and OTR show similar time courses as Sh-21 with no indication of substrate inhibition. The carbon recovery is fulfilled from the beginning to the end of the experiment, so no accumulation of metabolites was observed. The initial biomass concentration of Sh-24 (Fig. 4. 36) was lower compared with the other batches and the initial sodium acetate concentration was 3.35 g L^{-1}, the highest for all batches. Here, the nearly linear increase of the OTR and CTR is not accompanied by an equal increase of the biomass concentration. The biomass seems to be formed mainly in the last 1.5 h before substrate conversion is complete. All other data are in accordance with the other runs. The carbon recovery (not shown) decreased to 0.90 until the substrate was completely depleted. After that, a slightly increase of carbon recovery to 0.95 was observed.

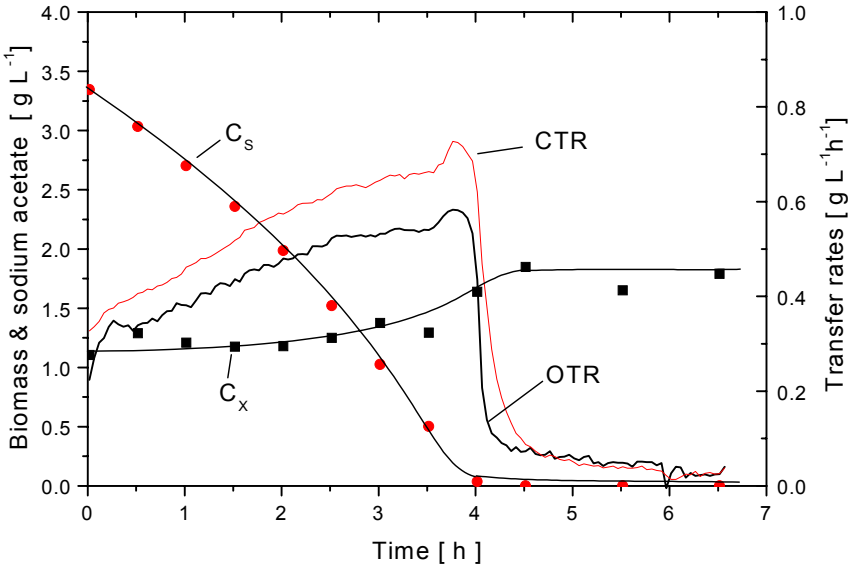

Fig. 4. 36. Experimental data for batch cultivation Sh-24 on sodium acetate.

In general, deviation of the carbon recovery, if any , is not so prominent as for phenol and benzoate, and it seems that intermediates are further metabolized to biomass and carbon dioxide.

Fig. (4. 37) shows the specific conversion rates of sodium acetate for the three batches. The calculated r_S values are much higher compared with benzoate and phenol. In addition, there seems to be an influence of the initial acetate concentration. In contrast to phenol, higher values were calculated for the batches with higher initial acetate concentration. If inhibition hampers growth at higher acetate concentrations can not be definitely stated. Although r_S is decreasing with increasing acetate concentration for batch Sh-23, no inhibition is observed for Sh-21 and Sh-24 up to an acetate concentration of 2.0 g L^{-1}.

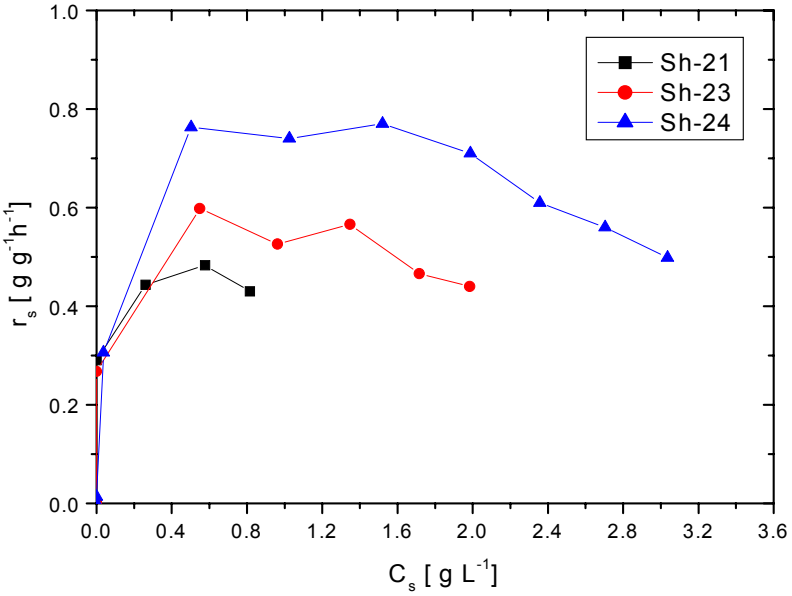

Fig. 4. 37. Specific substrate consumption rates versus sodium acetate concentration for the three batch cultivations.

4. 2. 1. 4. 2. Wash-out cultivation

On sodium acetate, two wash-out experiments were carried out. The first experiment was started out of a steady state at D = 0.20 h^{-1} and a sodium acetate feed of 2.0 g L^{-1}. The dilution rate was switched to D = 0.43 h^{-1}, and the feed concentration was 2.0 g L^{-1} after the switch. The experimental data are shown in Figure (4. 38). Acetate accumulation started immediately, but the OTR, CTR, and the biomass concentration remained approximately constant during the whole experiment, decreasing only slightly. The initial biomass concentration (first data point) is most probably an outlier (data scatter), because the steady state biomass concentration, calculated from the yield and maintenance coefficients, should be only 0.73 g L^{-1}, which is an accordance with the other biomass data.

The carbon recovery (not shown) decreased to a minimum of 0.87 in the first hour, but increased again and reached fulfilment. If a biomass concentration of 0.73 g L^{-1} is used as starting point, the carbon recovery is fulfilled for the whole experiment.

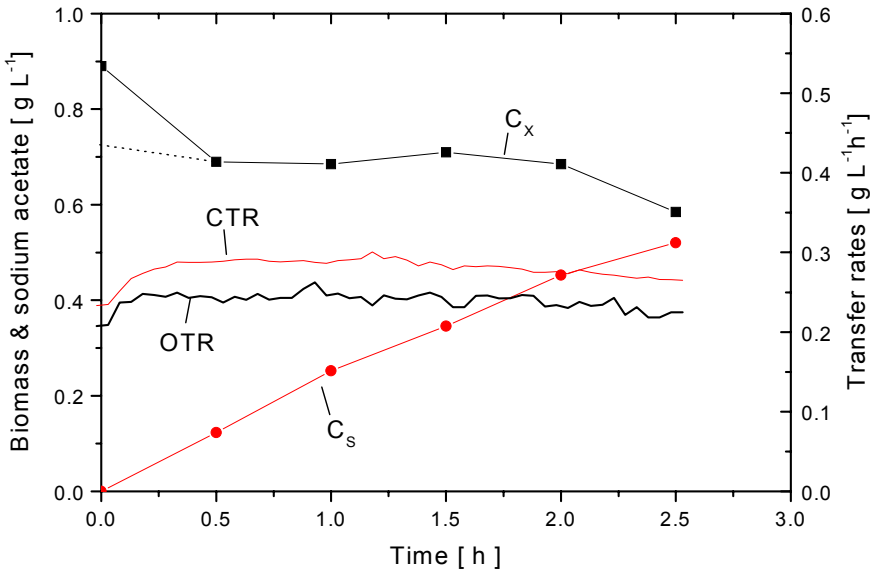

Fig. 4. 38. Experimental data of wash-out cultivation at D = 0.43 h^{-1} on sodium acetate.

Fig. (4. 39) show the specific acetate consumption rate and specific growth rate as a function of the sodium acetate concentration.

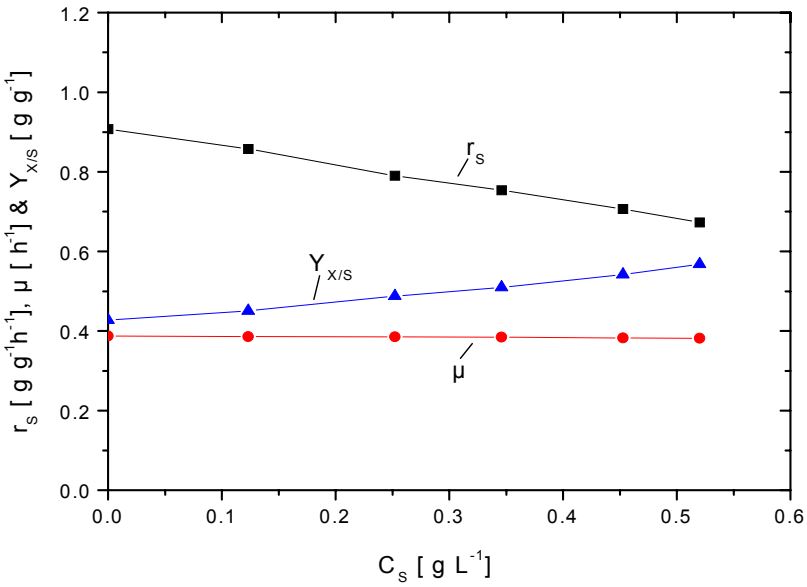

Fig. 4. 39. Specific growth rate and specific acetate consumption rate in addition to the biomass yield coefficient for the first wash-out cultivation on sodium acetate.

For calculation of μ, an initial biomass concentration of 0.73 g L^{-1} was used. r_S decreases steadily from 0.91 g g^{-1} h^{-1} to 0.76 g g^{-1} h^{-1}, whereas μ decreases only slightly. μ was estimated to approximately 0.38 h^{-1} during the whole experiment, being lower than the dilution rate (0.43 h^{-1}). The decrease of r_S with increasing sodium acetate concentration may indicate substrate inhibition, but if there is any inhibition, the inhibitory effect is not very significant.

Although sodium acetate is accumulating rapidly, it is evident that the cell can grow with a specific growth rate of 0.38 h^{-1}. A nearly constant biomass in combination with sodium acetate accumulation means that the biomass yield must increase after the dilution rate step. The yield was calculated as $Y_{X/S}$ = 0.37 g g^{-1} before the D-step, and $Y_{X/S}$ = 0.43 g g^{-1} after the D-step. The yield then increased to 0.57 g g^{-1} during the time course of the experiment (Fig. 4. 39). This is an astonishing result, because the calculated $Y_{X/S}$ is higher than the true growth value obtained from steady states. It is commonly assumed that the maximum yields are obtained under substrate limiting conditions at steady state.

Here, the situation is different compared with phenol. For phenol, the anabolic routes adapt more slowly than the catabolic routes, which lead to accumulation of metabolites. The specific growth rate of sodium acetate in this run increased immediately after the step change to 0.38 h^{-1}, a value close to the new dilution rate of 0.43 h^{-1}. The specific acetate consumption rate also increased to a high value, but considerably lower than the corresponding value for the new dilution rate. It seems that growth is limited by substrate conversion. This is also supported by the fulfilled carbon recovery. A slow adaptation (increase of μ and r_S) was not observed in this experiment. Perhaps a time span of 2.5 hours was too short. Adaptation in the phenol wash-out experiment started after approximately 2.8 hours (Fig. 4. 18). If the duration of this experiment was too short for adaptation to take place, and if the growth was limited by the catabolic enzymes, then the maximum specific growth rate might be even higher than 0.38 h^{-1}. Therefore it would be interesting if a higher yield than in a steady state can be performed for a longer time and if μ can be further increased. Therefore, a second wash-out experiment was carried out and observed for a longer time (Fig. 4. 40).

The second run was started out of a steady state at D = 0.20 h^{-1}, but using a higher feed concentration of 3.36 g L^{-1}. The dilution rate was shifted-up to 0.48 h^{-1}, and the feed concentration changed slightly to 3.24 g L^{-1} after the step increase. As in the previous experiment, accumulation of sodium acetate started immediately. After 2 h, a maximum was passed, and after that the sodium acetate concentration decreased again. After 4.5 h, complete consumption was re-obtained. The OTR and CTR increased steadily from the start of the D-step up to the point where complete sodium acetate comsumption was re-obtained.

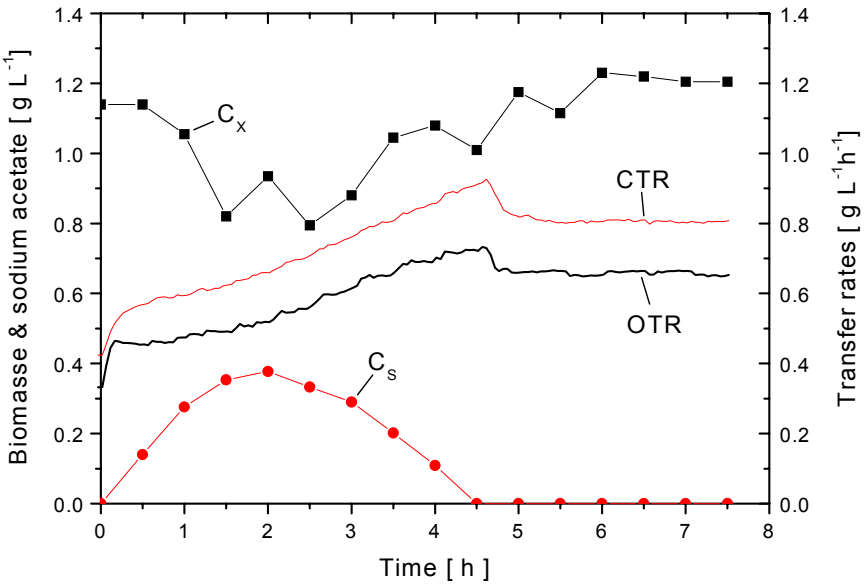

Fig. 4. 40. Experimental data of the second wash-out cultivation on sodium acetate at D = 0.48 h^{-1}.

The time course indicated that the cells performance is constantly and steadily improving until substrate limiting conditions are re-obtained. The biomass data correspond to the other data. Firstly, the biomass is decreasing due to wash-out conditions, but eventually the cell are getting fitter and the biomass increases again. After 5 h, all data becomming constant which indicates approach of a steady state. The data from this (stationary) part of the experiment fitted perfectly to the other steady states as was already shown in the steady state chapter.

This means that wash-out was not obtained at dilution rates up to 0.48 h^{-1}. The biomass was difficult to measure during the two acetate wash-out experiments because the cells showed a tendency to build flocs under these conditions. This effect was not observed at any other experiment. Therefore, the biomass data of this run were smoothed before calculating the specific growth rate.

Fig. (4. 41) shows the calculated specific rates and the biomass yield. After the D-step, the specific rates seem to be constant for some time, then to increase up to a maximum value (adaptation), which were constant until the accumulated sodium acetate is totally used up or removed by convective flow, respectively. The plateau values of μ and r_S may therefore be assumed as maximum specific rates: μ_{max} = 0.60 h^{-1} and r_S =1.68 g g^{-1} h^{-1}. The biomass yield remains below the steady state value during the whole experiment. A prolonged time range with a higher yield then the steady state value was not observed in this experiment.

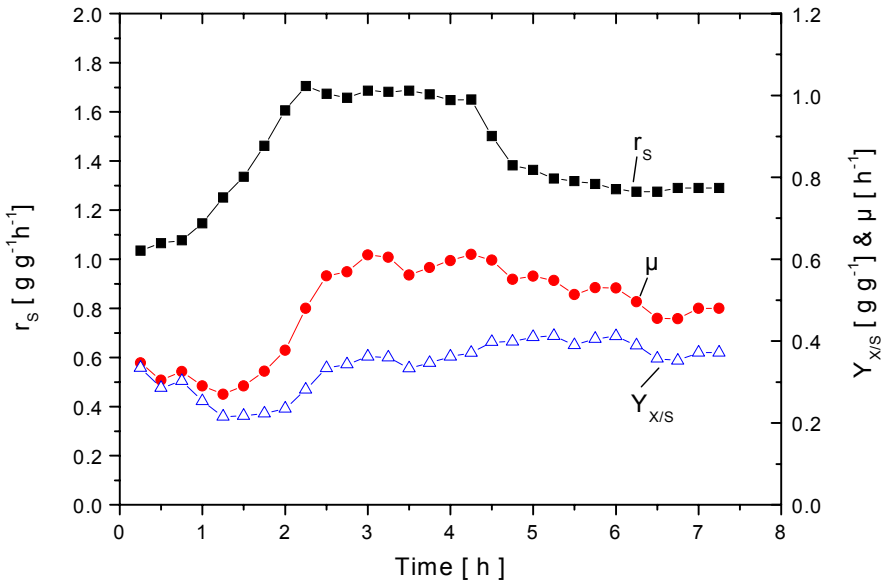

Fig. 4. 41. Specific growth rate, specific substrate consumption rate and biomass yield of the second wash-out cultivation on sodium acetate at D = 0.48 h^{-1}.

Although the cells can grow at higher rates as 0.48 h^{-1}, the enzyme level in the cells is too low to switch immediately to the new conditions. The result is a temporary wash-out condition, so the biomass decreases and sodium acetate accumulates. After a short time which is needed to initiate formation of new enzymes, the enzyme level in the cell increases by transcription / translation and μ and r_S start to increase up to their maximum values. When substrate consumption exceeds substrate feed, the accumulation maximum is passed and the substrate concentration decreases again. When all the accumulated substrate is depleted, the specific rates drop to the corresponding steady state values.

A similar behaviour was observed by Duboc (1997) for acetate conversion using *Saccharomyces cervisiae*. Duboc observed that, after a step change, the growth rate immediately increased to a new higher value, but not as high as the expected theoretical value of the new state. After some time, the growth rate increased and finally reached the new steady state value. A decoupling of anabolism and catabolism in transient phases, leading to different time constants was also observed by Duboc et al. (1998).

The carbon recovery (not shown) was fulfilled during the whole experiment except of a small deviation between 1 and 2.5 hours. The biomass data showed a higher scatter in this region, so it is not quite clear if this deviation is significant.

In general, growth on sodium acetate did not lead to wash-out up to dilution rates of 0.48 h^{-1}. Only temporary wash-out was observed, which was overcome by adaptation of the cells. The expected maximum specific growth rate should be approximately 0.60 h^{-1}. Both "wash-out" experiments showed different results. In the first one at D = 0.43 h^{-1} and C_{S0} = 2.0 g L^{-1} the cell growth immediately adapted to the new conditions, whereas in the sceond run at D = 0.48 h^{-1} and C_{S0} = 3.24 g L^{-1} the cells needed an adaptation phase. It is not clear, whether the higher D or C_{S0} or both is the main reason caused for this discrepancy. In both runs, an adaptation phase of the acetate conversion leaded to temporary accumulation of sodium acetate. The high yields of the first run were not observed in the second. Moreover, slowly adaptation in the second run led to a new steady state which corresponded well with the other steady states. As for benzoate, substrate inhibition is not very significant.

4. 2. 1. 5. Conclusion of the single substrate cultivations

The carbon balances in steady state cultivations with phenol, benzoate and acetate as sole sources of carbon, were fulfilled or very close to 100%. Deviations of the carbon recoveries, however, occurred in situations where the cells responded to dynamic changes of the growth conditions during transient experiments or batch experiments, if phenol or benzoate was used as substrate. If acetate was applied as carbon source, no or only light deviation from fulfilment was observed. Therefore, substrate carbon is stored during transient phase on phenol or benzoate in metabolic intermediates. This can be reserve material in the bacterial cells, true dead end metabolites, or precursors of biosynthetic routes. It is also possible that substrate carbon is removed from the metabolic route by oxidation reactions if compounds are piled up due to a bottleneck.

Under certain conditions, the cells are able to grow at considerably higher metabolic rates. Accumulation of metabolites as demonstrated by the deviation of the carbon recovery is not the regulatory factor, because the growth rate on phenol was limited to 0.30 h^{-1} at steady states (Schröder et al. 1997) where the carbon recovery was fulfilled, but higher growth rates were observed in the wash-out experiment which was accompanied by a deviation of the carbon balance. The regulatory factor may be the substrate concentration itself, because growth rates higher than 0.30 h^{-1} on phenol were only obtained at substrate concentrations below the detection limit. If the critical dilution rate is approached by a stepwise increase of the dilution rate, even small steps might temporaryly increase the substrate concentration, which will then lead to a limitation of the growth rate. Also, metabolic products, formed in small concentrations in the cell, can act as regulatory factor. Performance of the cells is also dependent on culture history. Following the theory of Kovárová-Kovar and Egli (1998), the cells adapt to their present environmental conditions by varying the kinetic and stoichiometric parameters. This was especially shown for the benzoate cultivations. The higher the growth rate in the preceding chemostat, the higher was the initial substrate consumption rate in the following adjusted batch. The dependence of the culture history could be expressed as a variation of the maximum specific substrate consumption rate as a function of the dilution rate of the preceding chemostat.

For phenol, a additional influence factor adds to the complexity of the system. A phenol batch, whose initial phenol concentration was only marginally higher compared with another phenol batch, showed a considerably slower conversion, despite the fact that the dilution rate of the preceding chemostat was twice as high. This additional influence factor is contradictory to the influence of the culture history (initial growth rate), concealing its influence in the case of the two batches. The nature of this additional influence factor is yet unknown. It seems not very likely that the slightly higher initial phenol concentration, 0.92 g L^{-1} instead of 0.90 g L^{-1} is the reason herefore, although phenol is known to destabilize the cell membrane.

During transient phases after a step up of the dilution rate or the substrate concentration, the cells adapt to the new situation after a short lag phase by increasing their specific rates. The time constant of this adaptation is in the range of several hours, therefore it is highly likely a genetic effect (increasing the enzyme level in the cells). The time constants of enzyme regulation are in the range of milliseconds. The adaptation is linked to the influence of culture history, resulting in a variation of kinetic parameters. The time constants of the adaptation, especially for phenol and benzoate, are different, resulting in an uncoupling of catabolism and anabolism. This uncoupling is most probably the reason for the accumulation of metabolites or the deviation of the carbon recovery, respectively.

In general, all three substrates can be completely degraded if applied individually as sole source of carbon and energy. For phenol and benzoate, metabolic intermediates accumulate during dynamic stages. Phenol is subject to a strong substrate inhibition, benzoate and sodium acetate show only a slight inhibition. The maximum growth rate on phenol was 0.47 h^{-1}. It seems likely that this value is also valid for benzoate, although the highest growth rate, which was experimentally obtained, was 0.30 h^{-1}. On sodium acetate, the maximum specific growth rate is about 0.60 h^{-1}.

4. 2. 2. Cultivation of substrate mixtures

In the previous chapters, growth and degradation kinetics of *Burkholderia cepacia* G4 was investigated at dynamic and stationary cultivation conditions for the three substrates applied individually as sole carbon source. It has also been shown that a substrate mixture of all three compounds is completely and simultaneously degraded under substrate limiting condition in a chemostat. The purpose of this chapter is to investigate interaction of substrates in substrate mixtures at dynamic and substrate surplus conditions, respectively. Especially of interest is if the toxic compound phenol is used up in the presence of less toxic compounds. Therefore, phenol was present in all mixtures. Mixtures of two substrates (binary) and others of three substrates (ternary) were used as sole source of carbon and energy in batch and A-stat experiments.

4. 2. 2. 1. Phenol-acetate

4. 2. 2. 1. 1. Batch cultivation

Using adjusted batch techniques, three batch experiments were carried out using a mixture of phenol and sodium acetate as carbon source. Different initial concentrations of both substrates were supplied to the reactor. All experiments were carried out after establishment of a steady state on a phenol-sodium acetate substrate mixture using a dilution rate of D = 0.10 h^{-1}. The culture history and the initial feed concentrations of the two substrates are given in Table (4. 9).

Table 4. 9. Culture history and initial feed concentrations of phenol and sodium acetate associated in a substrate mixture for the adjusted batch cultivations.

Experiments	Culture history				C_{S0}-batch	
		C_{S0}-chem. mixture				
	D-chem. 1/h	phenol g L^{-1}	sodium acetate g L^{-1}	C_X-chem. g L^{-1}	phenol g L^{-1}	sodium acetate g L^{-1}
Sh-27	0.105	0.8646	0.7114	1.430	0.5876	0.6127
Sh-28	0.105	0.9107	0.6969	1.530	0.4247	2.6043
Sh-29	0.107	0.9384	0.6926	1.395	0.7935	0.3497

For the first batch (Sh-27), comparable concentrations of phenol and sodium acetate were supplied. The experimental data of this batch are shown in Fig. (4. 42). It illustrates that phenol was completely consumed after 2.6 h, followed by acetate after 3.6 h from beginning.

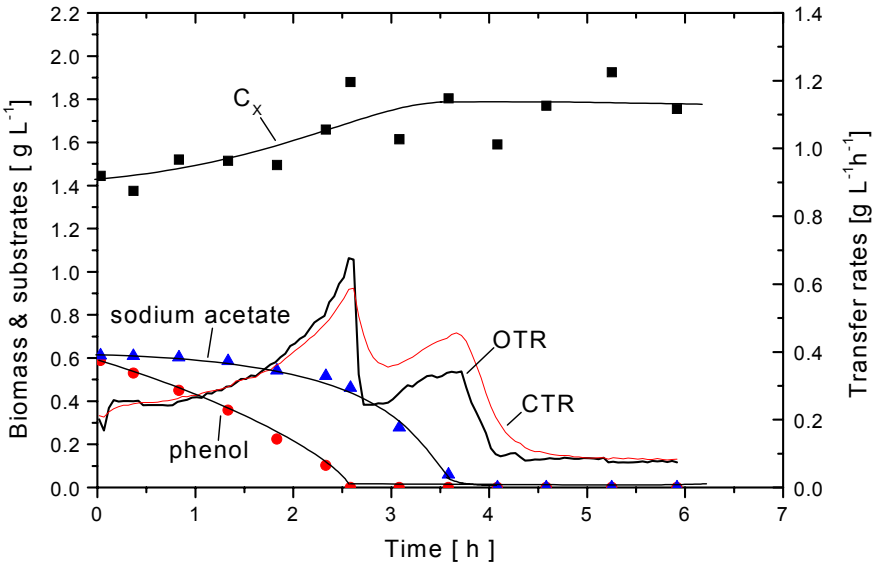

Fig. 4. 42. Experimental data for batch cultivation Sh-27 on a phenol-sodium acetate mixture.

It is evident that both substrates were metabolized simultaneously, indicating the the enzymes of both pathways were induced coordinately. However, sodium acetate conversion is strongly inhibited by phenol, which is the preferred substrate. Acetate conversion increases after phenol was completely used up at t = 2.5 h. Biomass increased from 1.44 to 1.80 g L^{-1} during this time period, which corresponds to a yield of 0.30 g g^{-1}, considerably lower compared with steady state growth for each substrate individually.

If there is a cross inhibition, especially if acetate inhibits phenol conversion, can not be definitely answered. Compared with batch cultivations on phenol alone, however, degradation of phenol is slower in this experiment. The initial phenol conversion rate of batch Sh-10 is 0.25 g g^{-1} h^{-1}, whereas in this experiment it is only 0.13 g g^{-1} h^{-1}. Initial acetate conversion is low. So, the anabolic routes, which tackle the combined metabolic flow from both substrates, are not subject to overload considering the initial specific rates in this experiment. It is therefore probable, that acetate inhibits phenol conversion, although it is not so evident from the progress curves in Fig. (4. 42).

The time courses of OTR and CTR show the typical substrate inhibition pattern as was obtained for the phenol batches. In general, two peaks were observed. The first results from conversion of both substrates together, but is mainly influenced by phenol degradation. After complete consumption of phenol, the transfer rates drop according to the reduced substrate conversion and show the slow linear increase which is characteristic for acetate conversion alone.

After complete conversion of phenol, the OTR drops immediately to its new reduced value, whereas the CTR decreases more slowly. This may be an indication of a decoupling of catabolism and anabolism, which was already observed in the individual substrate batches. A delayed anabolism leads to a slowly decreasing metabolic activity, during which carbon dioxide is formed. On the other hand, oxygen is not necessary for these reactions (Fig. 4. 42).

The carbon recovery (not shown) decreased slightly before reaching its minimum of 0.92 at the same time as phenol is completely used. After that, the carbon recovery data are nearly constant. This observation corresponds well with the individual substrate batches. It seems that the decoupling of anabolism and catabolism, which is most probably the cause for the metabolites accumulation, is initiated by the *meta* pathway or its products, respectively.

The second batch (Sh-28) was carried out to investigate the inhibitory effect of phenol on acetate in the presence of high concentration of sodium acetate in the mixture. Initial concentrations of 2.60 g L^{-1} sodium acetate and 0.42 g L^{-1} phenol were used for this purpose. The experimental data are plotted in Fig. (4. 43).

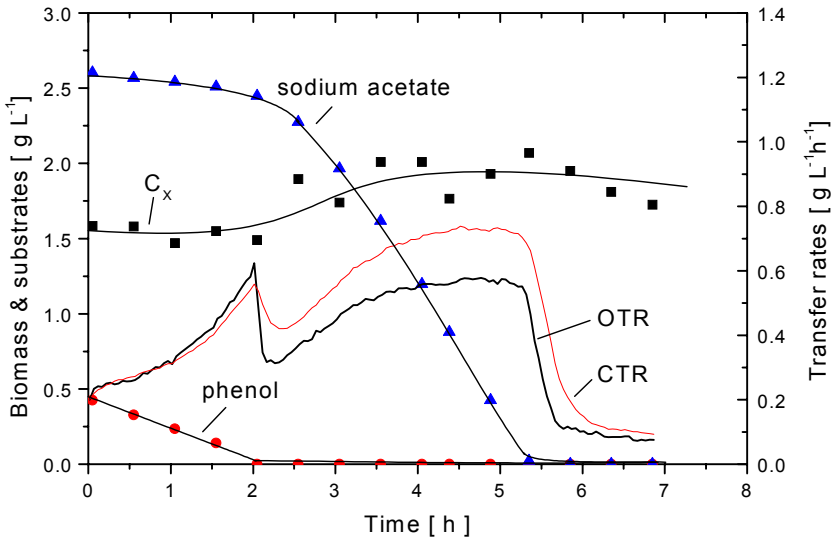

Fig. 4. 43. Experimental data for batch cultivation Sh-28 on phenol-sodium acetate mixture.

As in the previous run, simultaneous utilization was observed. After complete conversion of phenol, sodium acetate was consumed rapidly. Phenol was used up after 2 h, followed by sodium acetate after 5.8 h from the start. During oxidation of phenol, only a slow degradation of acetate was observed compared with its velocity after depletion of phenol, indicating a strong inhibition of phenol on acetate. On the other hand, phenol likewise seems to be influenced by of sodium acetate.

During the whole experiment, the biomass yield is lower compared with steady state cultivation. It should be pointed out here, that even low concentrations of phenol provoke a strong inhibition effect.

The time courses of OTR and CTR reflect the metabolic activity of the culture. They also demonstrate the difference in decoupling of metabolic routes. OTR and CTR differ considerably in the decrease after complete utilization of phenol, whereas the time courses are fairly parallel after complete utilization of acetate. The carbon recovery data (not shown) show the same pattern as for the previous run. A minimum value of 0.92 g g^{-1} was achieved after utilization of phenol at t = 2.0 h, which remains constant after that.

For batch run Sh-29, the initial phenol concentration was increased to 0.79 g L^{-1} in the presence of only 0.35 g L^{-1} sodium acetate. The experimental data are given in Fig. (4. 44). Although a much higher concentration of phenol compared with sodium acetate was used, it was the first substrate to be completely degraded after 3.7 h, following by acetate after 4.2 h. During this time, the biomass increased from 1.30 to 1.67 g L^{-1}, which corresponds to an overall yield of 0.32 g g^{-1}. During degradation of phenol, the inhibitory effect towards sodium acetate utilization is evident. The time courses of OTR and CTR show similar behaviour as observed in the previous runs.

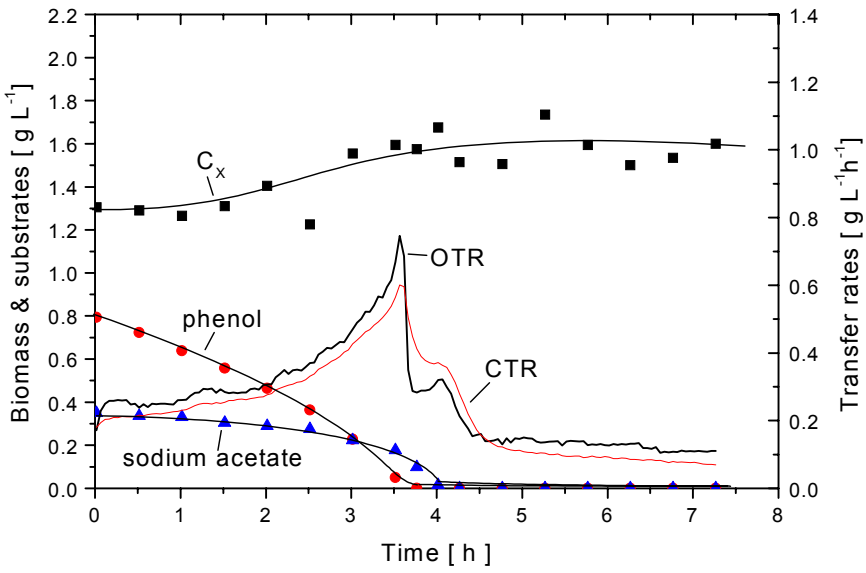

Fig. 4. 44. Experimental data for batch cultivation Sh-29 on phenol-sodium acetate mixture.

The carbon recovery (Fig. 4. 45) decreased slowly before reaching the minimum value of 0.85. As for the previous runs, carbon recovery data remained constant after degradation of both substrates, indicating formation of dead end metabolite.

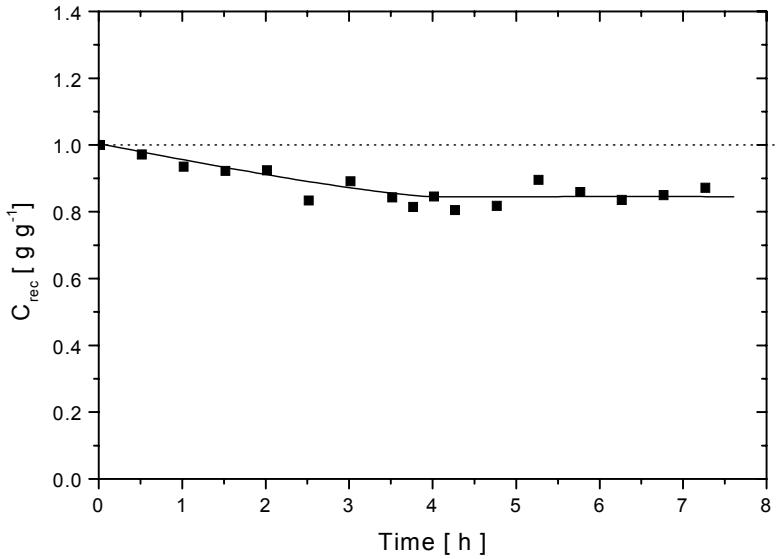

Fig. 4. 45. Carbon recovery data for batch cultivation Sh-29 on a phenol and sodium acetate.

Fig. (4. 46) compares the time courses of phenol (a) and sodium acetate (b) for the three batch experiments. For all experiments, the batch was started at an identical dilution rate of D = 0.10 h^{-1}, therefore the "culture history" was identical. For phenol, the time courses are fairly parallel and seem to be mainly influenced by the initial phenol concentration.

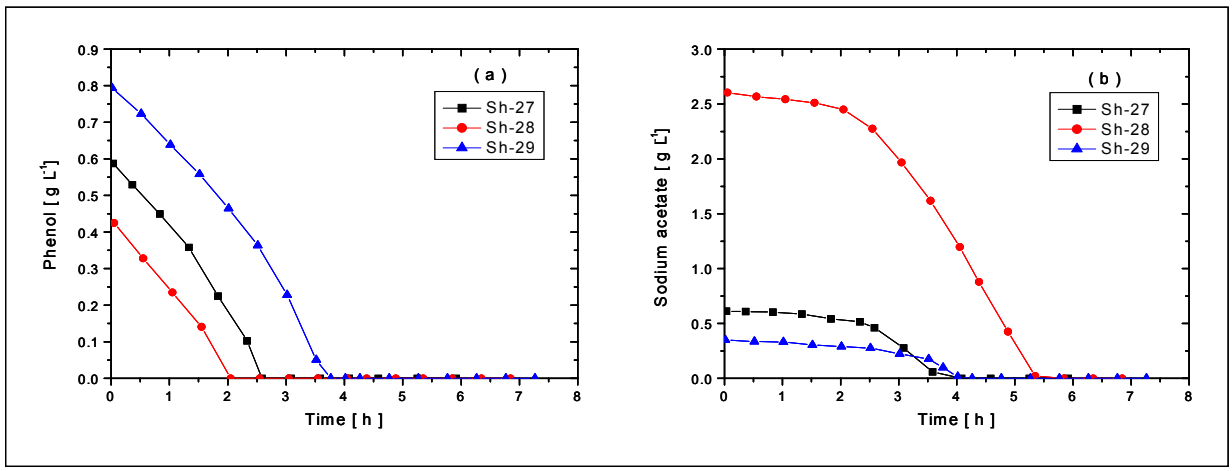

Fig. 4. 46. Comparison between the time courses of the three batch experiments: (a) for phenol and (b) for sodium acetate.

The time course of Sh-28 is slightly slower compared with the other experiments, revealing at least a slight inhibition of acetate on phenol degradation, because Sh-28 has the highest concentration of sodium acetate, absolutely and in relation to phenol. Conversely, degradation of sodium acetate was strongly inhibited, even at low phenol concentrations. Both substrates, phenol and acetate, were utilized simultaneously. This was also observed by Schmidt and Alexander (1985) by using *Pseudomonas acidovorans*. The authors were the first to demonstrate that aromatic pollutants can be utilized simultaneously with other compounds by pure cultures of bacteria.

Specific phenol consumption rates as function of the phenol concentration are plotted in Fig. (4. 47). The time courses for the three experiments do not fall together. The highest phenol conversion rates were achieved for the batch with lowest sodium acetate concentration, and the lowest r_S-values for the batch with the highest sodium acetate concentrations. This reveals at least a slight inhibition of sodium acetate on phenol conversion.

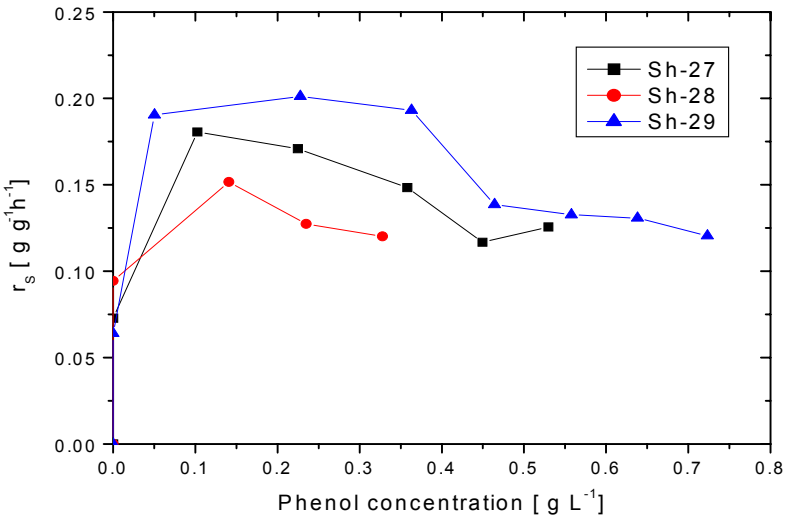

Fig. 4. 47. Specific phenol consumption rates as function of the phenol concentration for the batch experiments on a phenol-sodium acetate mixture.

Fig. (4. 48) shows a similar plot for the specific acetate consumption rate. During simultaneous utilization of both substrates, the r_S-values for sodium acetate were below 0.10 g g^{-1} h^{-1}. After complete conversion of phenol, r_S increases. An increase was also observed for the sodium acetate batch experiments (Fig. 4. 37), but not so prominent. Perhaps the toxic effect of phenol on the cell membrane permeability can be taken into account here (Keweloh et al. 1989 & 1990). The authors found an increase in the cell membrane permeability is in the presence of phenol, which reduced the lipid to protein ratios of the membrane compared with cells grown without phenol. This effect is slowly reversible after depletion of phenol.

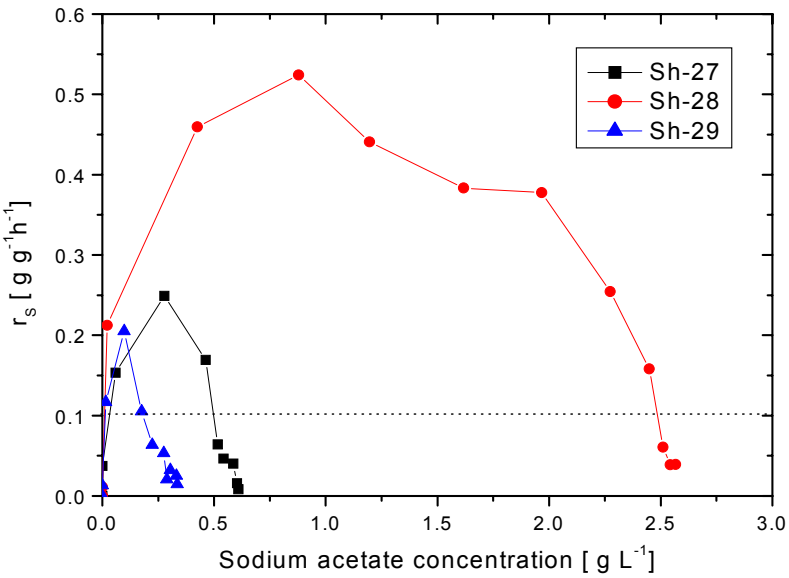

Fig. 4. 48. Specific acetate consumption rates as function of the sodium acetate concentration for the batch experiments on a phenol-sodium acetate mixture.

Fig. (4. 49) shows a comparison between single substrate and substrate mixture experiments for phenol (Fig. 4. 49a) and sodium acetate (Fig. 4. 49b). Experiments with similar initial substrate concentrations were selected. In both cases, the single substrate conversion is faster as is shown in the progress curves, and the specific consumption rates. Fig. (4. 49b) demonstrates the strong phenol inhibition on acetate utilization.

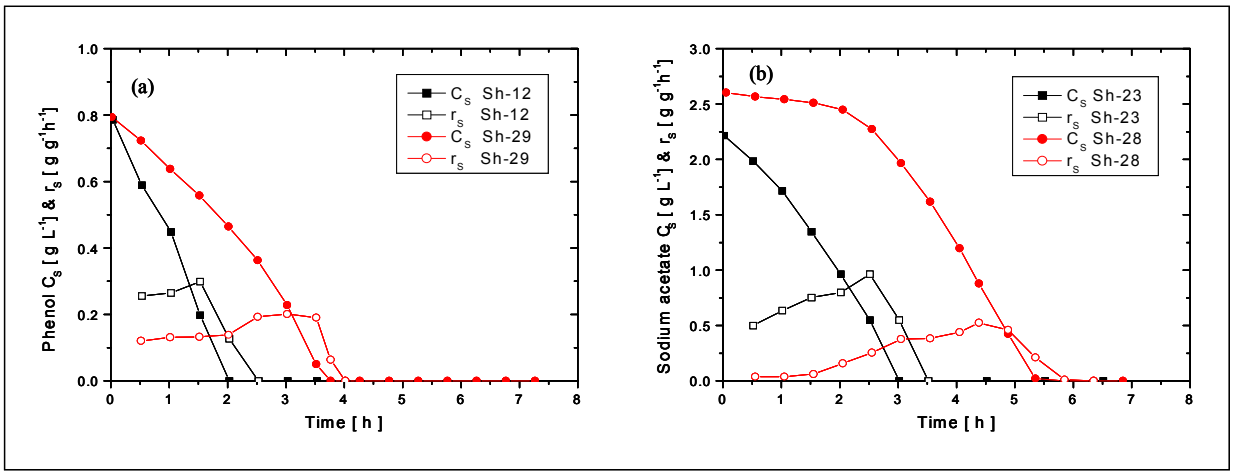

Fig. 4. 49. Comparison between degradation of a single substrate and of the same substrate in a mixture for phenol (a) and sodium acetate (b).

In general, the reduction of specific rates at simultaneous utilization of both substrates can be caused by two effects. Firstly, cross inhibition among both substrates in the mixture. This is based on the direct inhibition of the degrading enzyme of the first substrate by the second compound. If acetate has an affinity for the phenol-degrading enzyme, it binds to it. This binding prevents the mineralization of phenol, hence the phenol removal rate is reduced. Similarly, phenol acts on the acetate-degrading enzymes. A cross inhibitory pattern was also described for phenol and glucose by *P. putida* ATCC17514 (Wang et al. 1996). The authors observed that reduction of specific substrate utilization rates indicates that the two substrates are involved in a cross inhibitory pattern.

Based on regulation of the enzyme activity explained by Schlegel (1992), the cells may be contain sensitive systems to adjust activity of these enzymes, in addition to regulation of its levels. Although degradation of phenol and acetate are via different metabolic pathways, acetyl-CoA is the first common compound resulting from both pathways before the metabolic flow is directed towards the tricarboxylic acid (TCA) cycle. Acetyl-CoA may therefore be a bottleneck, which can lead to an overflow of the anabolic routes. Metabolites may pile up and act as inhibitors to reduce metabolic flow through the catabolic routes (product inhibition). Degradation of one mole phenol leads to two moles acetyl-CoA, but only one mole results from removal of one mole acetate. Metabolic flows were checked for experiment Sh-27 (Fig. 4. 42), revealing that the combined metabolic flow in this experiment was lower than metabolic flows achieved for single substrate experiments, so piling up of metabolites due to overflow is not very likely.

Nevertheless, regulatory compounds may be generated by different flows through single involved enzymes of the active pathways. Even low concentrations of regulatory compounds can act as inhibitors to block or reduce flow through catabolic pathways.

4. 2. 2. 2. Phenol-benzoate

Regarding the strong inhibition of phenol towards acetate uptake, phenol was the preferred substrate in a phenol-sodium acetate mixture. Compared with phenol, benzoate is less toxic, but is metabolized via the same degradation pathway with exception of the first enzymatic step. It is therefore interesting, whether phenol is also preferred over benzoate, i. e. if phenol acts as a strong inhibitor on benzoate.

4. 2. 2. 2. 1. Batch cultivation

Under adjusted batch conditions, three experiments using a mixture of phenol and benzoate as carbon source were carried out. As for the phenol-sodium acetate mixture, all cultivations were established from the same dilution rate of $D = 0.10\ h^{-1}$. The culture history and the initial concentrations of both substrates are given in Table (4. 10).

Table 4. 10. Culture history and initial feed concentrations of phenol and benzoate for the adjusted batch cultivations.

Experiments	Culture history				C_{S0}-batch	
	D-chem. h^{-1}	C_{S0}-chem. mixture		C_X-chem. g L^{-1}	phenol g L^{-1}	benzoate g L^{-1}
		phenol g L^{-1}	benzoate g L^{-1}			
Sh-30	0.099	0.8117	0.6703	1.031	0.6128	0.5144
Sh-31	0.098	0.7302	0.6805	0.740	0.4159	1.0859
Sh-32	0.097	0.6389	0.7472	0.970	0.7299	0.2952

As observed during degradation of benzoate as a sole source of carbon, a yellow colouration showed up in the reactor in all batch runs, indicating formation of 2-hydroxymuconic semialdehyde (2-HMS). This was also observed by Johnson and Stanier (1971), Ottow and Zolg (1974), Rogers and Readon (2000), Singirtsev et al. (2000) and Spain and Nishino (1987).

For the first batch (Sh-30), a mixture of 0.61 g L^{-1} phenol and 0.51 g L^{-1} benzoate were supplied to the reactor as carbon source. The experimental data as function of the time are shown in Fig. (4. 50).

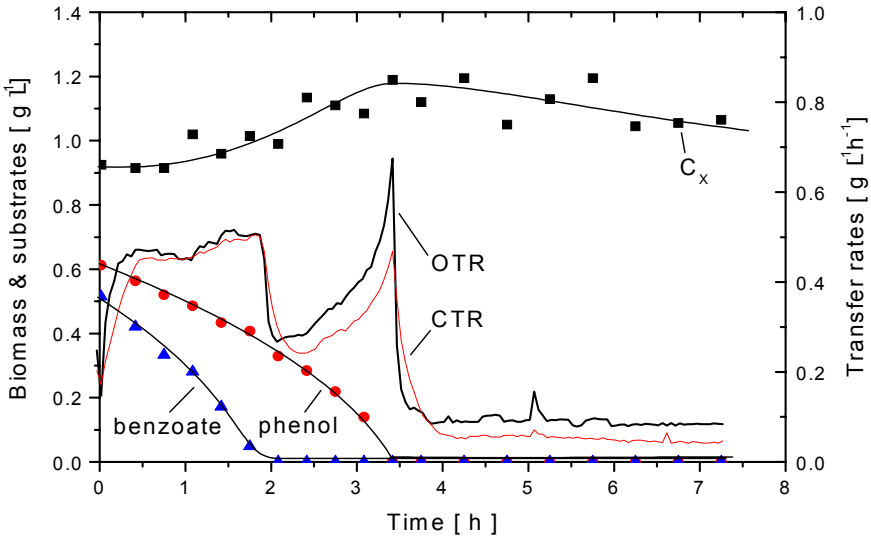

Fig. 4. 50. Experimental data of batch cultivation Sh-30 on phenol-benzoate mixture.

As observed for the phenol-sodium acetate mixtures, simultaneous utilization was obtained for the phenol-benzoate mixture with benzoate being the first substrate completely degraded. The time required to remove benzoate was 2.1 h, while phenol was consumed completely after 3.4 h from the start of the run.

During degradation of both substrates, biomass increased from 0.93 to1.19 g L^{-1}, which corresponded to an overall yield of 0.24 g g^{-1}, considerably lower than the steady state value. The time courses of OTR and CTR show different phases. In the first 2.0 hours, there is only a slight increase of the transfer rates. This phase resembles that of the benzoate batch cultivations. After depletion of benzoate, the rates decrease abruptly, and in the following 1.5 hours, they show the typical substrate inhibition pattern of phenol. From the time courses in the first 2.0 hours, there is no indication of a cross inhibition between both substrates.

The carbon recovery (Fig. 4. 51) gradually decreased and reached its minimum of 0.71at the same time as both substrates are completely used up. Here, the deficiency is considerably greater compared with the single substrate experiments or the phenol-sodium acetate mixture. After depletion of both substrates, the C_{rec} data are constant, indicating formation of dead end metabolites.

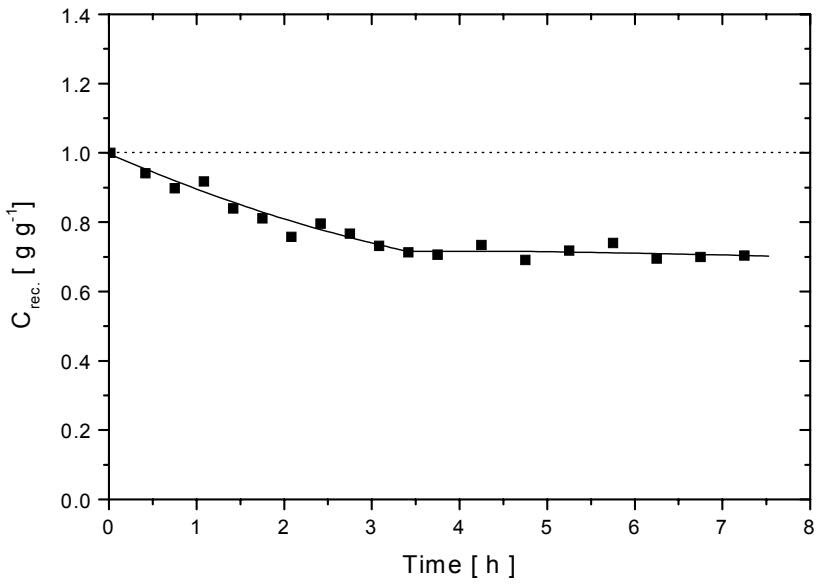

Fig. 4. 51. Carbon recovery for degradation of phenol-benzoate mixture in batch cultivation (Sh-30).

The great deficiency of the carbon recovery is probably due to a combinatory effect of both substrates.

For the second batch (Sh-31), a lower phenol concentration of 0.42 g L^{-1} and a higher benzoate concentration of 1.09 g L^{-1} were used. The experimental data are given in Fig. (4. 52). As expected from the first experiment, phenol and benzoate were utilized simultaneously in the mixture, and a yellow colouration appeared in the reactor.

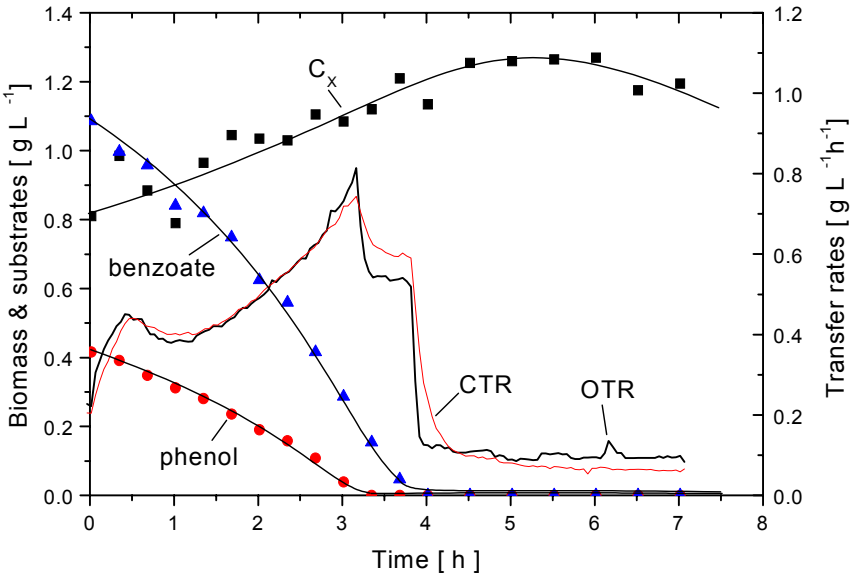

Fig. 4. 52. Experimental data of batch cultivation Sh-31 on phenol-benzoate mixture.

Here, phenol is the first substrate metabolized completely after 3.4 h, while benzoate was used up shortly after at 4.0 h. It should be noticed that a lower concentration of phenol took the same time to be used up compared with Sh-30. This result indicates that phenol degradation is probably reduced in the presence of a higher concentration of benzoate. The initial degradation rates depict the difference between both substrates, the high substrate inhibition of phenol. Although the initial concentration of benzoate is more than twice as high as phenol, the initial benzoate conversion rate is much faster. The biomass increased from 0.81 to 1.21 g L^{-1} in the first 4.0 hours, which corresponded to an overall yield of 0.27 g g^{-1}.

The time courses of OTR and CTR show a prominent peak at approximately t = 0.5 h. The reason here fore is not known. At the peak, the transfer rates have approximately the same value as during the first 2.0 hours of the previous batch experiment, so the peak is mainly caused by benzoate conversion. The time constant of this peak and the following minimum is too large for enzyme regulation (~ milliseconds), so its not very likely that this is an effect of competitive inhibition. It is well known that phenol alters the permeability of the cell membrane, so this may be the cause. If enzyme regulation causes the peak and minimum, then a product further down the pathway act as inhibitor. After the minimum, the transfer rates increase progressively, showing the effect of both phenol and benzoate degradation. As in the batch cultivations on the single compounds, the CTR is delayed during fast dynamic changes compared with OTR, indicating decoupling of catabolism and anabolism.

The carbon recovery (not shown) represents the same behaviour as in the previous batch. After reaching the minimum of 0.77 g g^{-1} at t = 4.0 h, the carbon recovery is constant for the rest of the cultivation.

An inhibitory effect of phenol against benzoate was not detected in the previous two experiments. In the third batch using this substrate composition, a high concentration of phenol was used together with a low concentration of benzoate (Fig. 4. 53).

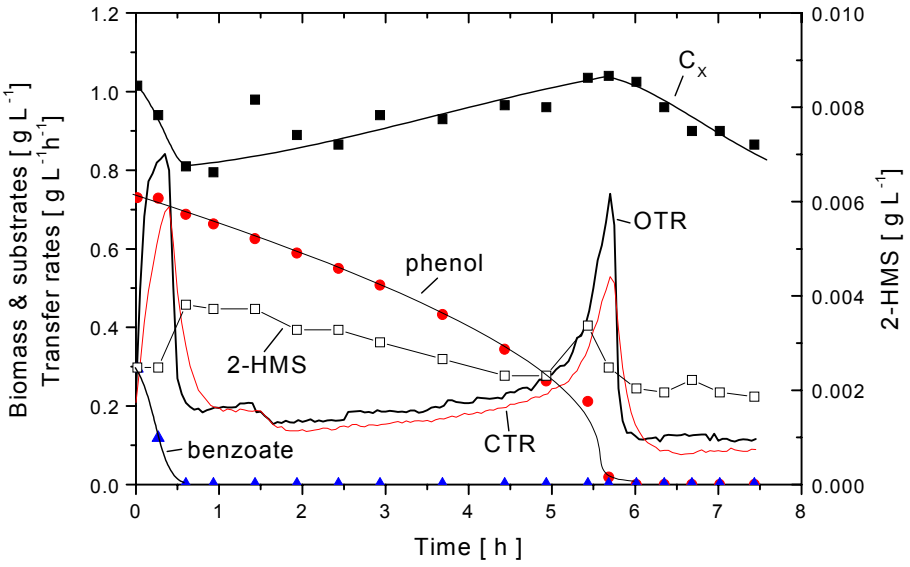

Fig. 4. 53. Experimental data of batch cultivation Sh-32 on phenol-benzoate mixture.

Because all cultivations using benzoate as single substrate or in a substrate mixture with the only exception of the chemostat on all three substrates showed the yellow colouration, the 2-HMS concentration was measured and plotted in Fig. (4. 53). Compared with the substrate concentrations, the 2-HMS concentrations are very low and do not count significantly for the deficiency of the carbon recovery.

As for the two previous batches, there was a simultaneous utilization of both substrates observed. The time required to degrade the mixture of 0.73 g L^{-1} phenol and 0.30 g L^{-1} benzoate was 6.0 h and 0.5 h, respectively. Although a high phenol concentration was used, benzoate was the first substrate completely degraded. The data do not reveal an inhibitory effect of phenol against benzoate degradation.

The rapid utilization of benzoate corresponded well with the prominent peak of OTR and CTR at the start of the cultivation. At this period, decrease of biomass from 1.02 to 0.81 g L^{-1} may be caused by an increase of the permeability of the cell membrane succeeded by an increase of the respiratory activity of the cytoplasmic membrane in the presence of phenol (Keweloh et al. 1989 & 1990). After the complete conversion of benzoate, OTR and CTR show the inhibitory pattern of phenol. During degradation of phenol, biomass increased again to 1.04 g L^{-1}.

A carbon recovery deficiency was also observed for this batch. The carbon recovery (not shown) decreased to a much lower value (0.68 g g^{-1}) compared with the other runs.

Although this is mainly a phenol cultivation with a small amount of benzoate added, the carbon recovery showed a higher deficiency than in comparable phenol batches. The same was observed for the other two experiments using the substrate mixture. The deficiency was also higher than in batches on single substrates. The reason is most probably an effect of decoupling combined with initial conversion rates. In general, conversion rates on benzoate are higher than on phenol because of the not so prominent substrate inhibition. All experiments using the phenol-benzoate substrate combination were carried out from a chemostat at D = 0.10 h^{-1}. Compared with the benzoate batch originating from the same dilution rate, Sh-19 (Fig. 4. 23), the sum of the initial specific substrate consumption rates in the mixture is higher than for the benzoate batch. Whereas the benzoate batch (Sh-19) has an initial specific rate of 0.26 g g^{-1} h^{-1}, the sum of the initial conversion rates in the mixture are 0.35, 0.35 and 0.67 g g^{-1} h^{-1}, respectively. Especially Sh-32, who has the highest sum, also shows the highest deviation in the carbon recovery.

It can therefore be concluded, that the higher the initial rates, the more prominent will be the decoupling, which results in a higher deviation of the carbon recovery. On the other hand, the piled up metabolites are directed towards biosynthesis after substrate depletion only in small amounts, so that the carbon recovery resembles formation of true dead end metabolites. The reason for the non-use of the accumulated metabolites for biosynthesis can be many fold, lack of energy after substrate depletion or chemical oxidation because of instability for example. Compared with the phenol-acetate batches, chemical oxidation of the accumulated compounds is more likely than lack of energy, because the carbon recovery did not recover during conversion of acetate after phenol was completely used up (batch, Sh-28).

To investigate if there is any cross inhibition between phenol and benzoate, the initial specific substrate consumption rates were calculated separately for the two components (Table 4. 11).

Table 4. 11. Initial specific substrate consumption rates for phenol and benzoate of the batch cultivations in their mixture.

Experiments	Initial r_S g $g^{-1}h^{-1}$	
	phenol	benzoate
Sh-30	0.13	0.22
Sh-31	0.11	0.24
Sh-32	0.07	0.60

Because all experiments have been carried out from the same dilution rate of the preceding chemostat, the culture history is identical. The specific phenol consumption rates of Sh-30 and Sh-31 are nearly identical. The initial benzoate concentration of Sh-31 is twice as high as that of Sh-30 (Table 4. 10), therefore there is no inhibition of benzoate on phenol consumption detectable. Sh-32 has the highest initial benzoate consumption rate, but also the highest initial phenol concentration (Table 4. 10). Therefore, there is also no inhibition of phenol on benzoate conversion detectable.

In general, phenol and benzoate are degraded via the *meta* cleavage route. This was supported by the yellow colouration of the culture suspension in benzoate cultivation. It was shown that 2-HMS is not responsible for the large deficiency of carbon recovery during degradation of phenol-benzoate mixtures. Appearance of this colour in cultivations of benzoate and its mixture with phenol, but not with phenol alone, may indicate induction of other enzymes in the *meta* cleavage pathway. Therefore, phenol and benzoate are probably degraded via two different enzymes (isoenzymes) of the *meta* cleavage pathway. Accumulation of 2-HMS is, in general, influenced by the activity of two enzymes of the *meta* pathway, catechol-2,3-dioxygenase who catalyses formation of 2-HMS, and the following enzyme catalysing conversion of 2-HMS to 2-hydroxymuconate. Although conversion rates of benzoate in batch are higher than those of phenol, it is unlikely that the second enzyme is rate limiting due to higher metabolic flow in the upper part of the pathway, because the yellow colour was also observed at steady states on benzoate with low dilution rates.

4. 2. 2. 2. 2. A-stat cultivation

Under dynamic conditions (up shifts), it has been demonstrated that the biological system is subjected to decoupling of catabolism and anabolism, accumulation of metabolites, and slow adaptation of the specific conversion and growth rates. This applies for batch cultivations and wash-out experiments. It is therefore questionable if maximum specific growth and consumption rates can be estimated from these cultivation techniques. The A-stat, on the other hand, provides quasi stationary conditions.

If the acceleration factor is small enough that adaptation effects do not delay culture performance, then the A-stat should be well suited for a slow approach to maximum culture performance, unhampered by adaptation effects.

With phenol, an A-stat cultivation resulted in a critical dilution rate of D = 0.48 h^{-1}. Therefore it can be assumed that this is the true maximum value. On benzoate, only a wash-out experiment has been carried out, which resulted in a maximum specific growth rate of 0.30 h^{-1}. On the other hand, identical values of μ_{max} were calculated from the wash-out and the A-stat on phenol. Therefore it seems that under wash-out conditions, at least for phenol and benzoate, the maximum performance of the cells can be obtained from wash-out experiments.

An A-stat experiment was carried out using a mixture of phenol and benzoate. The experiment was started out of a steady state at D = 0.20 h^{-1} with feed concentrations of 0.88 g L^{-1} phenol, and 0.68 g L^{-1} benzoate, respectively. The acceleration factors of the medium, phenol and benzoate were calculated individually, resulting in an overall value of 0.048 h^{-2}. The experimental data are shown in Fig. (4. 54).

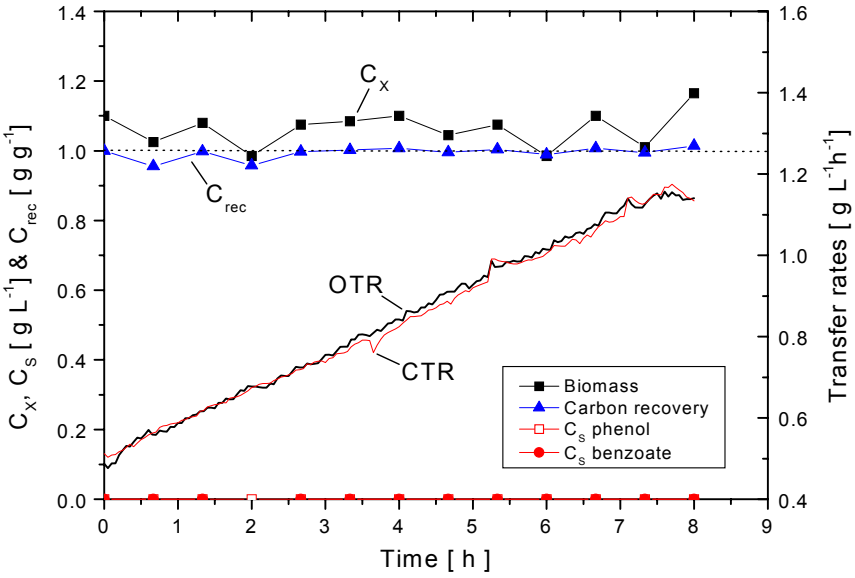

Fig. 4. 54. Experimental data of A-stat cultivation on a substrate mixture of phenol and benzoate.

Based on the overall acceleration factor, the dilution rate was increased linearly and reached 0.59 h^{-1} when the experiment was terminated. The time courses of OTR and CTR also increased linearly, indicating the enhancement of the metabolic activity. The biomass is constant, in spite of a slight decrease of the phenol and benzoate feed concentrations to 0.77 and 0.63 g L^{-1}, respectively.

The biomass concentrations correspond well with the theoretical values calculated by yield and maintenance coefficients. So the yield in this experiment is the same as in the chemostat experiments. Performance of the cells is constantly improving as the dilution rate increases. No substrate was detected in the medium. It means that the culture has not reached its maximum metabolic activity. Therefore, the cells can grow and metabolize the substrate mixture up to dilution rates of 0.59 h^{-1}, a value considerably higher than on phenol alone (0.48 h^{-1}, Fig. 4. 19).

As is shown in Fig. (4. 54), the carbon recovery is fulfilled over the whole experiment. This is, together with the biomass yield, a further indication that the culture conditions resemble steady state conditions.

The critical dilution rate, leading to wash-out, is equal or higher than 0.59 h^{-1}, and therefore considerably higher than in the A-stat on phenol. The second substrate increases the wash-out point. Although phenol and benzoate should be metabolized by the same pathway, the molar yield coefficients were estimated not to be identical. If a simple reaction mechanism according to Fig. (4. 55) is assumed, the highest dilution rate, at which a steady state can be achieved, is given if the overall substrate intake rate into the reactor does not exceed the maximum overall reaction rate, or

$$D_{crit.}\,((C_{S\,0\,(p)} - C_{S\,(p)}) + (C_{S\,0\,(b)} - C_{S\,(b)})) = (r_{S\,max\,(p)} + r_{S\,max\,(b)})C_X \qquad [4.\,23]$$

with

$$C_X = Y_{X/S\,(p)}\,(C_{S\,0\,(p)} - C_{S\,(p)}) + Y_{X/S\,(b)}\,(C_{S\,0\,(b)} - C_{S\,(b)}), \qquad [4.\,24]$$

equation [4. 23] leads to:

$$D_{Crit.} = \frac{(r_{S\,max\,(p)} + r_{S\,max\,(b)})(Y_{X/S\,(p)}\,(C_{S\,0\,(p)} - C_{S\,(p)}) + Y_{X/S\,(b)}\,(C_{S\,0\,(b)} - C_{S\,(b)}))}{(C_{S\,0\,(p)} - C_{S\,(p)}) + (C_{S\,0\,(b)} - C_{S\,(b)})} \qquad [4.\,25]$$

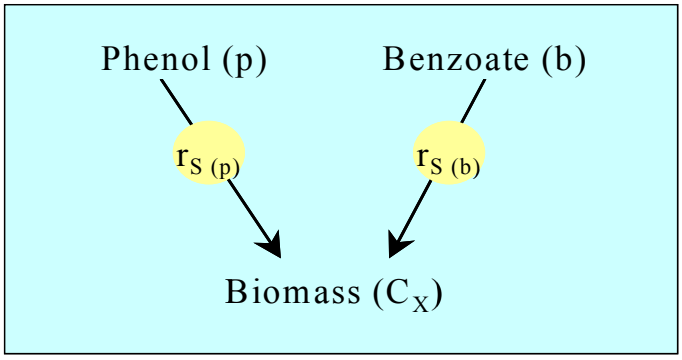

Fig. 4. 55. Simple reaction mechanism of phenol and benzoate conversion to biomass.

Here it was assumed that no accumulation of metabolites takes place, or that there is no overload of the anabolic flow if both catabolic flows are at their maximum rate. If both compounds have to same biomass yield, then the critical dilution rate is simply given by:

$$D_{crit.} = (r_{S\,max\,(p)} + r_{S\,max\,(b)})Y_{X/S} \qquad [4.\,26]$$

The dilution rate, at which the first of the two compounds will start to accumulate in the bioreactor, can be calculated accordingly:

$$D_{crit.\,(p)} = r_{S\,max\,(p)} (Y_{X/S\,(p)} C_{S\,0\,(p)} + Y_{X/S\,(b)} C_{S\,0\,(b)}) / C_{S\,0\,(p)} \quad [4.\,27]$$

or

$$D_{crit.\,(b)} = r_{S\,max\,(b)} (Y_{X/S\,(p)} C_{S\,0\,(p)} + Y_{X/S\,(b)} C_{S\,0\,(b)}) / C_{S\,0\,(b)} \quad [4.\,28]$$

if phenol or benzoate appears first. The ratio between both expressions can be used to calculate which compound will appear first:

$$D_{crit.\,(p)} / D_{crit.\,(b)} = r_{S\,max\,(p)} C_{S\,0\,(b)} / (r_{S\,max\,(b)} C_{S\,0\,(p)}) \quad [4.\,29]$$

The chemostat faces therefore three different phases:

1) $D < D_{i,\,crit.}$: steady state, both substrates are completely mineralized.

2) $D_{i,\,crit.} < D < D_{crit.}$: steady state, one compound is completely mineralized, the other concentration of the other compound and the biomass are function of the dilution rate (i is phenol or benzoate).

3) $D > D_{crit.}$: wash-out.

For the A-stat on phenol and benzoate, benzoate is the first substrate to accumulate: $D_{crit\,(p).}/D_{crit.\,(b)} = 1.19$.

If maintenance is not taken into account, benzoate is assumed to appear at $D = 0.72\ h^{-1}$.

Because the experiment was terminated at $D = 0.59\ h^{-1}$, no accumulation was observed. The experimental data also show, that the assumption, that the anabolic flow was not overloaded, is valid up to dilution rates of $0.59\ h^{-1}$.

4. 2. 2. 3. Phenol-benzoate-acetate

In cultivations of substrate mixtures composed of two compounds, phenol was utilized simultaneously in the presence of sodium acetate or benzoate, respectively. It was also shown, that phenol and sodium acetate exhibit a cross inhibition on each other. The inhibitory effect of phenol on sodium acetate was severe , whereas only slight inhibition of sodium acetate on phenol was observed. No inhibitory action, on the other hand, was observed between phenol and benzoate. The purpose of this chapter is to investigate degradation of a mixture of all three compounds, and in addition, to evaluate mutual influences between benzoate and acetate. To achieve this goal, batch and A-stat cultivations were carried out. The preceding chemostat was performed on all three substrates.

4. 2. 2. 3. 1. Batch cultivation

All experiments were carried out after steady states were established at a dilution rate of D = 0.10 h^{-1}. History of the culture and initial concentrations of substrates are given in Table (4. 12).

As observed for the phenol-benzoate mixtures, the yellow colouration showed up in the reactor during all cultivations, indicating, as mentioned, conversion of catechol via catechol-2,3-dioxygenase (*meta* route) to 2-HMS, and accumulation of this compound.

Table 4. 12. Culture history and initial concentrations for substrate mixtures of phenol, benzoate and sodium acetate at adjusted batch conditions.

Experiments	Culture history					C_{S0}-batch		
	D-chem. h^{-1}	C_{S0}-chem. mixture			C_X-chem. g L^{-1}	phenol g L^{-1}	benzoate g L^{-1}	sodium acetate g L^{-1}
		phenol g L^{-1}	benzoate g L^{-1}	sodium acetate g L^{-1}				
Sh-33	0.109	0.9328	0.5432	0.8522	1.055	0.4394	0.3981	0.4420
Sh-34	0.112	0.9617	0.5832	0.8874	1.235	0.3756	0.3018	1.9041
Sh-35	0.112	0.9916	0.5631	0.9051	1.275	0.6537	0.3107	0.3976

The first batch (Sh-33) was carried out by using similar initial concentrations for all substrates, approximately. The experimental data are given in Fig. (4. 56). It shows, that degradation of all substrates started immediately, indicating simultaneous utilization. Benzoate was the first substrate completely utilized after 0.35 h. Although similar initial concentrations were used, degradation of phenol took 2.82 h compared with 2.18 h for sodium acetate. It was also noticed that the degradation rate of phenol increased rapidly after complete conversion of sodium acetate. The data clearly show the inhibitory influence of sodium acetate on phenol.

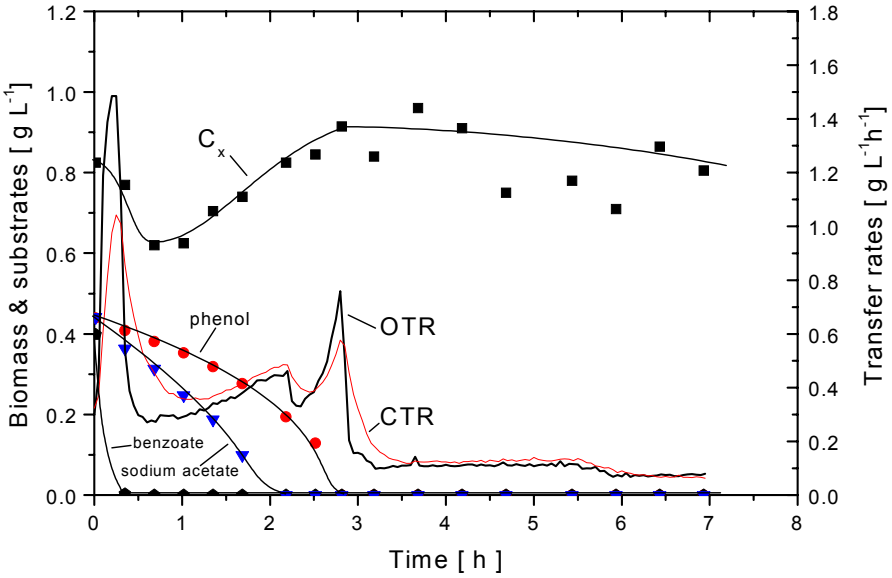

Fig. 4. 56. Experimental data of batch Sh-33 for cultivation on a substrate mixture of phenol, benzoate and sodium acetate.

The time courses of OTR and CTR show a prominent peak followed by a sharp decrease after degradation of benzoate. A conjunction between the rapid conversion of benzoate and OTR and CTR is obvious. At this time period, the biomass decreased from 0.83 to 0.62 g L^{-1}. During degradation of phenol and acetate, the biomass increased to 0.92 g L^{-1} which corresponds to a yield of 0.77 g g^{-1}. After complete removal of all substrates, the biomass decreased slightly.

The carbon recovery (Fig. 4. 57) decreased rapidly after inoculation and reached its minimum value of 0.70 at the same time as benzoate was completely used. After that, C_{rec} remained constant. It indicates formation of dead end metabolite. The high conversion rate of benzoate lead to a prominent deviation of the carbon recovery, where during phenol degradation no further deviation was observed. This is in contrast to all other batches on phenol, where always an increasing deviation was observed as long as phenol was utilized.

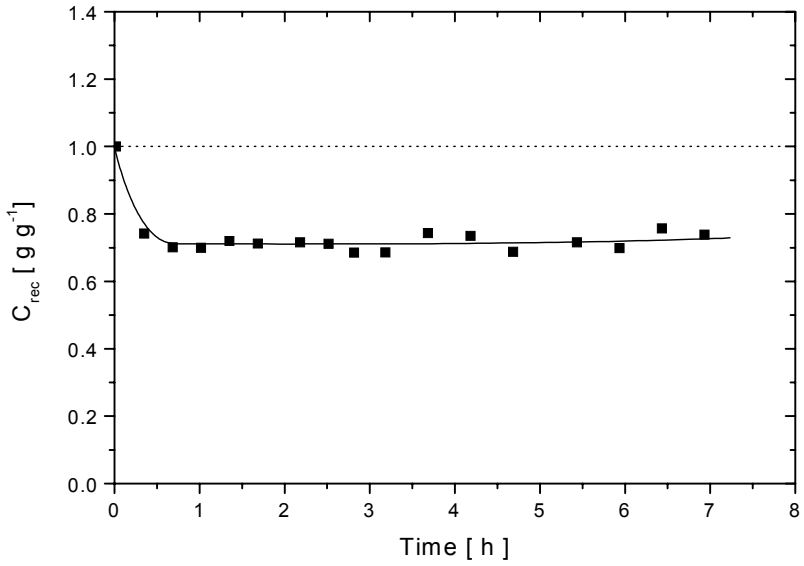

Fig. 4. 57. Carbon recovery data for batch cultivation Sh-33 on a substrate mixture of phenol, benzoate and sodium acetate.

In order to investigate any inhibitory effect of sodium acetate on benzoate, a second batch experiment was carried out using a high concentration of sodium acetate, 1.90 g L^{-1}, and low concentration of phenol and benzoate, 0.38 g L^{-1} and 0.30 g L^{-1}, respectively. The experimental data are shown in Fig. (4. 58). The data show that benzoate is also consumed rapidly, whereas 2.27 h and 2.93 h were needed to completely mineralize phenol and acetate, respectively. As for the previous batch, all substrates are utilized simultaneously.

As in the previous experiment, the biomass decreased at the beginning from 1.15 to 0.82 g L^{-1}, and the OTR and CTR showed a similar peak coupled with the degradation of benzoate. Biomass increased further to 1.31 g L^{-1} during oxidation of phenol and sodium acetate. After that, the time courses of the biomass and the transfer rates decreased.

Even at high sodium acetate concentrations, benzoate is rapidly metabolized, revealing no indication of inhibition of sodium acetate on benzoate. On the other hand, the strong inhibition of phenol on acetate conversion is not so obvious in this experiment.

The carbon recovery (not shown) shows a sharp decrease during benzoate utilization before gradually reaching its minimum value of 0.73 at the same time all substrates were depleted completely. As for the previous batch, the carbon recovery refers to formation of dead end metabolites.

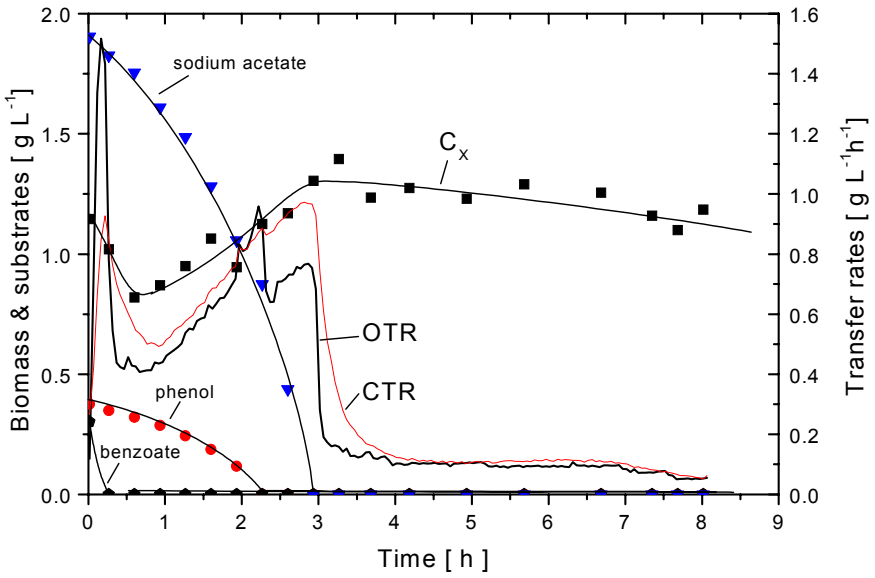

Fig. 4. 58. Experimental data of batch cultivation Sh-34 on a substrate mixture of phenol, benzoate and sodium acetate.

The third batch (Sh-35, Fig. 4. 59) was carried out using a high phenol concentration of 0.65 g L^{-1} in the presence of 0.31 g L^{-1} benzoate and of 0.40 g L^{-1} sodium acetate. As expected, benzoate was the first substrate consumed completely after about 0.18 h. The time required to degrade both of sodium acetate and phenol were 1.85 h and 4.77 h, respectively. Simultaneous utilization was achieved, and, like the two previous batches, decrease of biomass concentration from 1.07 g L^{-1} to 0.80 g L^{-1}after starting the experiment was observed. The time courses of OTR and CTR show a similar pattern as in the other two experiments.

The carbon recovery data (not shown) reached a minimum of 0.74 g g^{-1} and remained constant after that. Reduction of the metabolic activity after complete removal of all substrates leads to decrease of biomass and transfer rates.

Compared with the phenol-sodium acetate mixtures, the phenol inhibition on acetate conversion is not so prominent in batch with three substrates (Fig. 4. 44 and Fig. 4. 59), whereas benzoate conversion is faster than in the phenol-benzoate experiments and for benzoate alone. The third substrate alters the substrate conversion pattern considerably. Benzoate is the preferred substrate, and phenol seems to be the less preferred. In the phenol-sodium acetate mixture, phenol is preferred due to its strong inhibition on sodium acetate, whereas in the phenol-benzoate mixture, no interference between both substrates was observed.

The ternary mixture also differs from the other experiments by the biomass decrease during benzoate uptake, which was not observed in the other experiments, with exception of Sh-32, which also showed a rapid benzoate uptake in the presence of phenol. All compounds are utilized simultaneously, but the mechanism is complex and quite unclear.

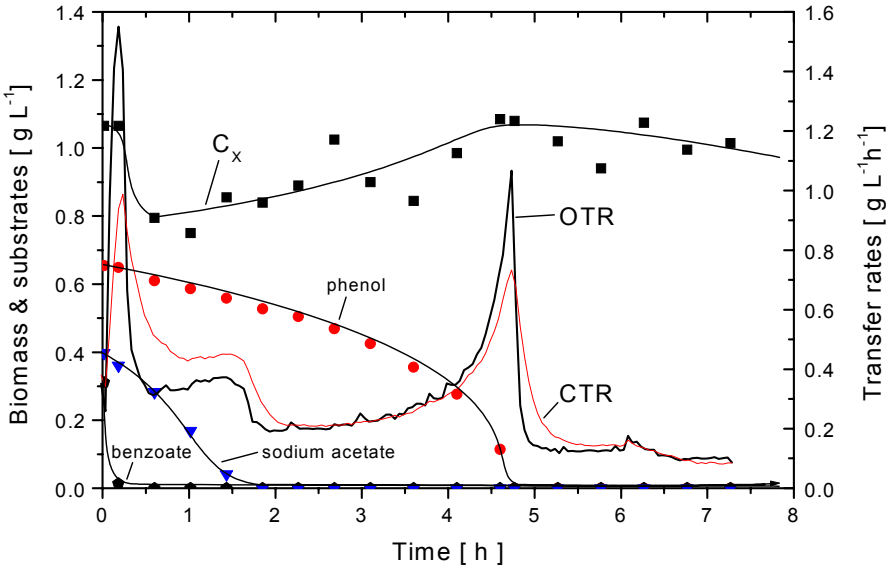

Fig. 4. 59. Experimental data of batch cultivation Sh-35 on a substrate mixture of phenol, benzoate and sodium acetate.

It has been shown, that in phenol-sodium acetate substrate mixtures a cross inhibition between both substrates occur. The phenol conversion is only slightly hampered in the presence of sodium acetate, whereas the sodium acetate conversion is strongly inhibited by phenol. On the other hand, phenol and benzoate did not show any indication of inhibition in phenol-benzoate substrate mixtures. Therefore, a similar inhibition pattern should be expected in mixtures of all three substrates. The experimental data, however, show a different behaviour. Benzoate conversion rates in the mixture are higher than for benzoate alone, and the inhibition of sodium acetate by phenol seems not prominent in the mixtures of three substrate batches. This is illustrated in Fig. (4. 60) and Fig. (4. 61).

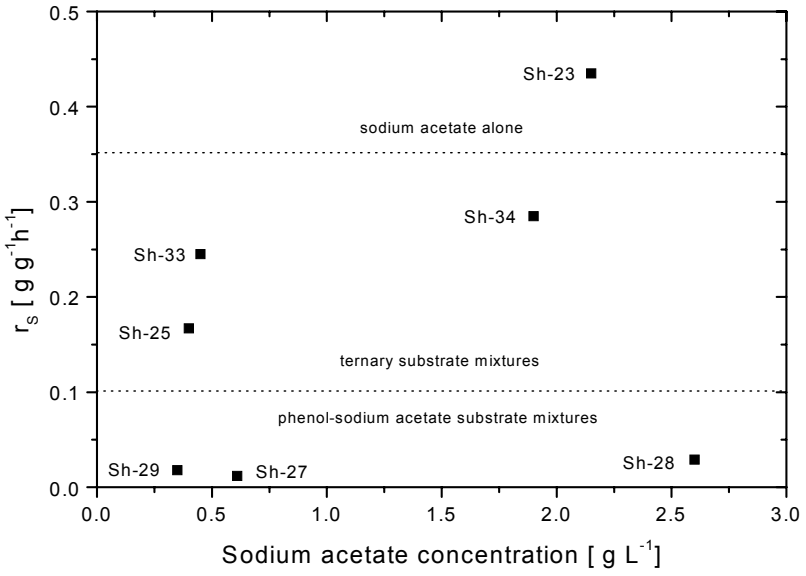

Fig. 4. 60. Initial specific sodium acetate consumption rates as function of the sodium acetate concentration for different batch cultivations.

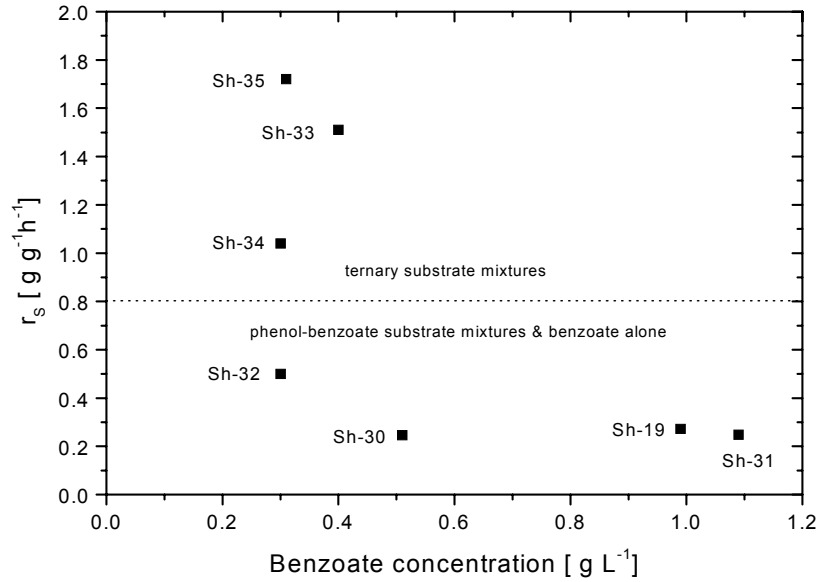

Fig. 4. 61. Initial specific benzoate consumption rates as function of the benzoate concentration for different batch cultivations.

Fig. (4. 60) shows the initial specific acetate consumption rates as function of the sodium acetate concentration, Fig. (4. 61) the corresponding plot for benzoate. Because of the influence of culture history, only batches performed out of the same dilution rate of the preceding chemostat (D ~ 0.10 h^{-1}) were compared. Fig. (4. 60) shows that all initial acetate conversion rates can be classified in three categories. The first category contains the batch on sodium acetate alone, which resulted in the highest conversion rate of 0.44 g g^{-1} h^{-1}. The second category contains all initial specific rates derived from the ternary substrate mixtures. The data range between 0.15 and 0.30 g g^{-1} h^{-1}. The third category contains the values of the phenol-sodium acetate substrate mixtures. All specific rates are below 0.03 g g^{-1} h^{-1}. It seems evident that the strong inhibition of sodium acetate by phenol is partly neutralized in the presence of the third substrate benzoate. The initial specific benzoate consumption rates in Fig. (4. 61) can be classified in two categories. All data, with exception of the mixtures containing all three substrates, are below 0.50 g g^{-1} h^{-1}. For the three substrate mixtures, the specific rates could not be calculated exactly because benzoate was already completely used up at the first sample after the initial sample. Therefore, the data given in Fig. (4. 61) are conservative, representing the lower limit. The specific rates in the three substrate batches might be considerable higher. Nevertheless, the data are all higher than 1.0 g g^{-1} h^{-1}, clearly showing that benzoate conversion is higher compared with the benzoate-phenol mixtures. A comparison with the single substrate batch is not possible because the experiment Sh-19, which was carried out at D ~ 0.10 h^{-1}, had a considerably higher initial benzoate concentration than in the mixture of all three substrates. Therefore, there seems to be a positive influence of sodium acetate on benzoate conversion and vice versa, leading to higher acetate and benzoate conversion rates, respectively, in the ternary substrate mixtures.

4. 2. 2. 3. 2. A-stat cultivation

Two A-stat cultivations were carried out to investigate growth on the three substrate mixture under dynamic but substrate limiting conditions. To estimate any influence of the acceleration factor, both experiments were performed at different acceleration factors. In the first run, an overall factor of 0.031 h^{-2} was used, in the second run a twice as high factor of 0.062 h^{-2}. Because of the slow acceleration, the first experiment was observed up to dilution rate of 0.48 h^{-1}, the second one up to 0.69 h^{-1}. Table (4. 12) show the acceleration factors for both experiments, and Fig. (4. 62) the experimental data of the first A-stat.

Table 4. 13. Experimental acceleration rates (a-factors) for each component in the reactor and range of the dilution rates used for the A-stat cultivations for the ternary substrate mixture.

Experiments	Acceleration rates (a-factors) [h^{-2}]					Dilution rates [h^{-1}]	
	medium	phenol	benzoate	sodium acetate	overall	D_0	D
A-stat 1	0.02129	0.00440	0.00212	0.00281	0.03062	0.238	0.483
A-stat 2	0.04187	0.01019	0.00474	0.00552	0.06232	0.251	0.687

The first A-stat experiment (Fig. 4. 62) was carried out starting from a steady state at D = 0.24 h^{-1}.

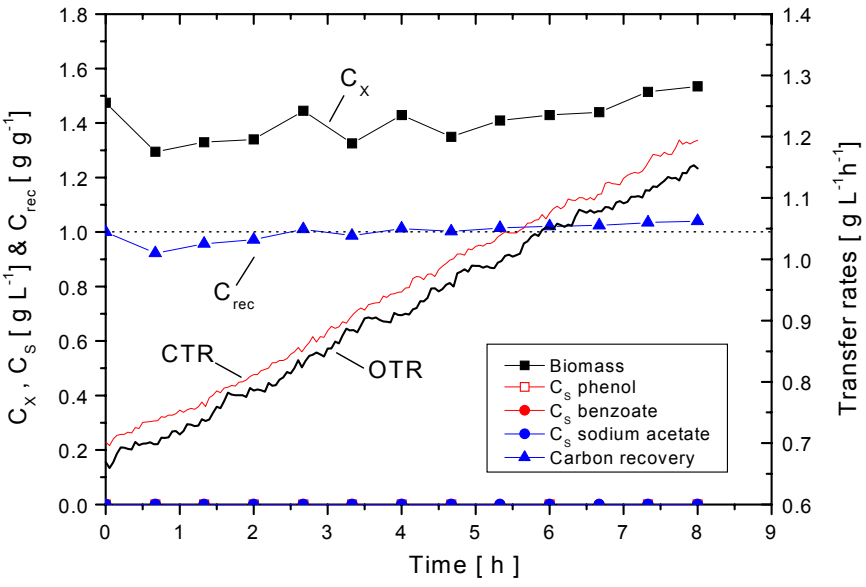

Fig. 4. 62. Experimental data of the first A-stat cultivation on a substrate mixture of phenol, benzoate and sodium acetate.

The experiment was started with feed concentrations of 0.86 g L^{-1} phenol, 0.65 g L^{-1} benzoate and 0.88 g L^{-1} sodium acetate. The dilution rate was increased linearly using an overall acceleration factor of a = 0.031 h^{-2}. Based on this factor, the dilution rate was observed up to 0.48 h^{-1} when the experiment was terminated. The data show that no substrate accumulation was detected in the medium over the whole time. The time courses of OTR and CTR increased also linearly, indicating increase of the metabolic activity. Although the feed concentrations decreased slightly to 0.81, 0.62 and 0.87 g L^{-1} for phenol, benzoate and sodium acetate, respectively, the biomass concentration is constant. No wash-out occurred under these conditions. The initial biomass concentration (first data point) corresponds well with the theoretical steady state biomass calculated by yield and maintenance coefficients. At the end of the experiment, the culture has not reached its maximum metabolic activity, which was expected from the results of the A-stat on phenol and benzoate.

As for true steady states, the carbon recovery is fulfilled, indicating a complete conversion of all substrates simultaneously.

Fig. (4. 63) shows the calculated specific growth and overall substrate consumption rates as function of the dilution rate. Both rates (μ and r_S) are approximately parallel, indicating a constant yield during the experiment.

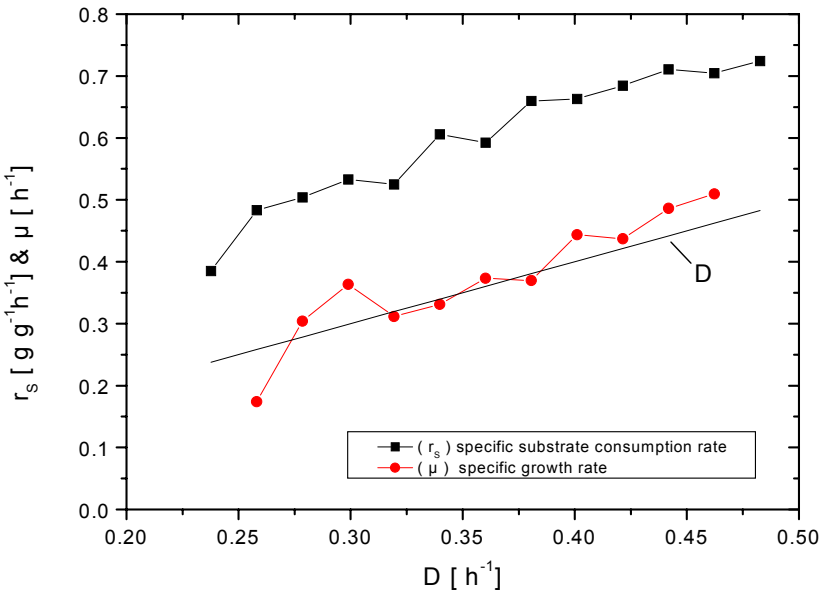

Fig. 4. 63. Specific growth rate and specific substrate consumption rate as function of the dilution rate for the first ramp (A-stat) cultivation of the ternary mixture.

From the yield and maintenance coefficients derived from the single substrate steady states, the theoretical biomass (equation [4. 2]) and the theoretical CTR:

$$CTR = r_{CO2\,(p)}\, Y_{X/S\,(p)}\, C_{S\,0\,(p)} + r_{CO2\,(b)}\, Y_{X/S\,(b)}\, C_{S\,0\,(b)} + r_{CO2\,(s)}\, Y_{X/S\,(s)}\, C_{S\,0\,(s)} \qquad [4.\ 30]$$

were calculated and compared with the experimental data of the A-stat (Fig. 4. 64).

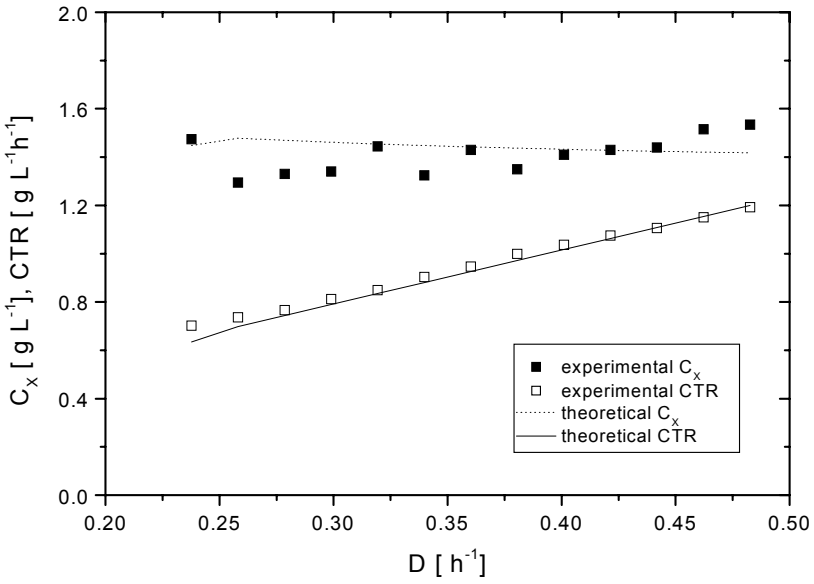

Fig. 4. 64. Theoretical values of CTR and the biomass concentrations compared with the experimental data as function of dilution rate for the first A-stat cultivation on the ternary substrate mixture.

Theoretical and experimental data are in good agreement, showing that the dynamic A-stat condition is comparable with true steady states for this acceleration factor, and also, that the stoichiometric parameters derived from single substrate steady states can be used to calculate biomass and carbon dioxide formation for an oligo substrate mixture under dynamic A-stat conditions.

The second A-stat cultivation was carried out using a higher overall acceleration factor of a = 0.062 h^{-2} (Fig. 4. 65). The experiment was started from a steady state at D = 0.25 h^{-1} with feed concentrations of 0.74, 0.63 and 0.82 g L^{-1} for phenol, benzoate and sodium acetate, respectively. A slight increase of the biomass concentration was observed, which was caused by an increase of the feed concentrations to 0.79, 0.64 and 0.83 g L^{-1} for phenol, benzoate and sodium acetate, respectively, over the course of the experiment.

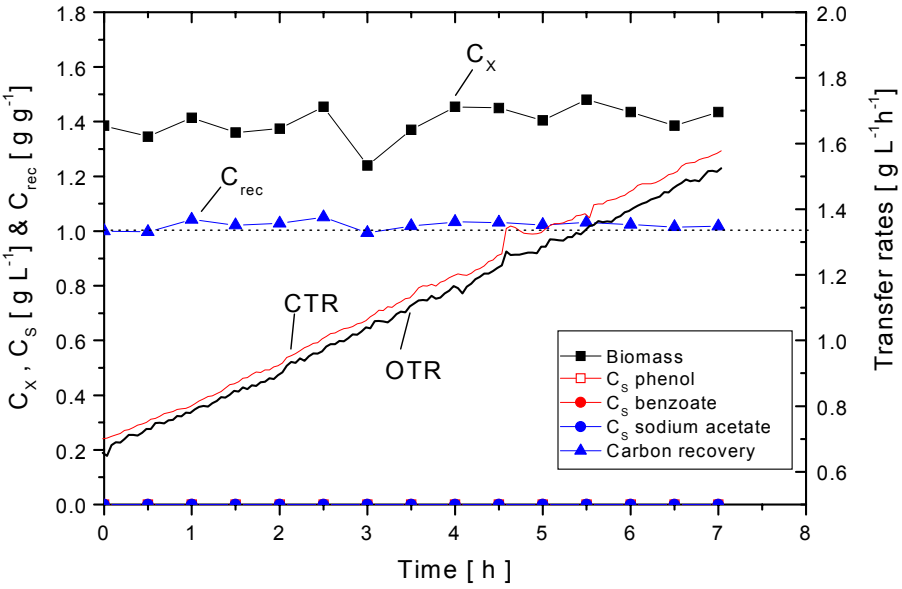

Fig. 4. 65. Experimental data of the second A-stat cultivation on the ternary substrate mixture.

Although a higher acceleration factor was used, and the dilution rate was increased up to 0.69 h^{-1}, no substrate accumulation was detected. The carbon recovery was fulfilled, and OTR and CTR increased linearly. A comparison between experimental and theoretical data (not shown), like for the previous A-stat, demonstrated comparability with steady state conditions. The acceleration in this experiment is therefore slow enough to allow the cells to adapt constantly to the changing conditions, and to prevent decoupling of catabolism and anabolism.

Fig. (4. 66) shows a comparison of all A-stat experiments carried out on single, dual and ternary substrate mixtures, and of all steady state data including the unstable steady state data of Schröder et al. (1997). For the phenol A-stat, which led to accumulation, only the data prior to phenol accumulation were plotted. All data with exception of the two A-stats using all substrates in the mixture fit the same straight line (Fig. 4. 66a). Because of the different yield coefficient of sodium acetate on the ternary mixture, the two A-stats show different r_S-values, and therefore they are plotted with the steady state data of the ternary mixtures in a separate Figure (Fig. 4. 66b). Again, all data in Fig. (4. 66b) fit the same straight line.

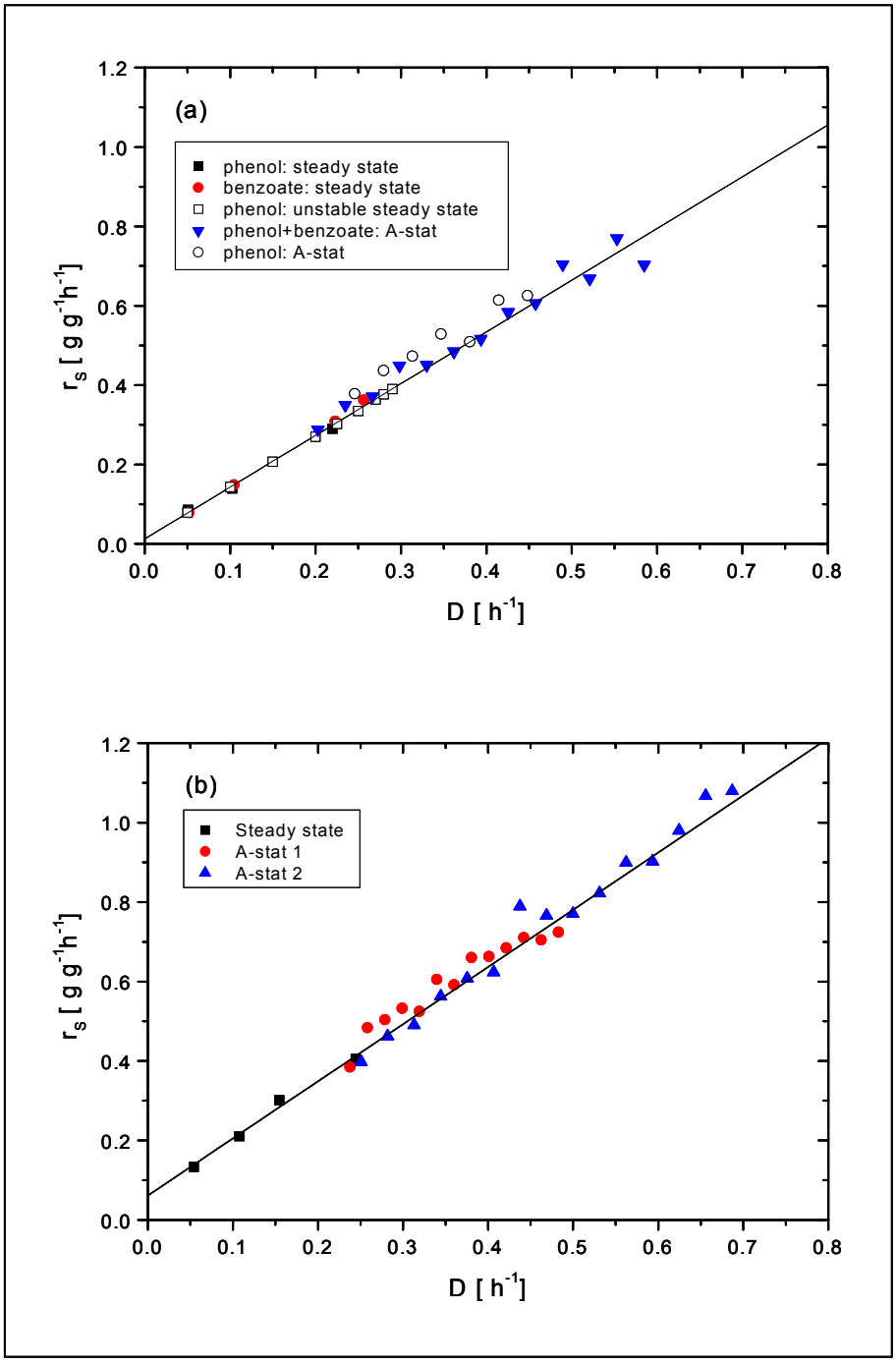

Fig. 4. 66. Specific substrate consumption rates calculated for different cultivation types compared with the fitting data of steady states: (a) for single and dual substrate mixture of phenol and benzoate, and (b) for the three substrate mixture as function of the dilution rate.

The data show, that A-stat cultivations can be described by the same set of stoichiometric parameters estimated from true steady states, even if the acceleration factor reaches 0.062 h^{-2}. Such a high acceleration factor was not used beforehand. Van der Sluis et al. (2001) concluded that an A-stat with an acceleration factor of 0.001 h^{-2} is attractive to be used instead of a chemostat cultivation when a strain of *Zygosaccharomyces rouxii* was cultivated in the presence of glucose under chemostat and A-stat cultivations. On the other hand, Paalme et al. (1995) used acceleration rates varying between 0.01 - 0.05 h^{-2} during A-stat cultivation of *Escherichia coli* growing on glucose.

It was shown for the A-stat on phenol and benzoate, that the dilution rate range, at which toxic phenol can be completely mineralized, can be extended if a second substrate is present. Based on the considerations for that experiment, the dilution rate at which the first of the three substrates will appear, was calculated for the second A-stat. The following maximum specific rates were used:

phenol:	$\mu_{max} = 0.48\ h^{-1}$,	$r_{Smax} = 0.605\ g\ g^{-1}\ h^{-1}$	(A-stat, Fig. 4. 19, and wash-out, Fig. 4. 16)
benzoate:	$\mu_{max} = 0.30\ h^{-1}$,	$r_{Smax} = 0.417\ g\ g^{-1}\ h^{-1}$	(wash-out, Fig. 4. 28)
sodium acetate:	$\mu_{max} = 0.60\ h^{-1}$,	$r_{Smax} = 1.569\ g\ g^{-1}\ h^{-1}$	(wash-out, Fig. 4. 40)

For benzoate, the values of the maximum specific rates are not yet clear. The highest specific growth and substrate uptake rates on benzoate were measured for the wash-out experiment, 0.30 h^{-1}, and 0.70 g g^{-1} h^{-1}, respectively (Fig. 4. 28). According to the steady state biomass yield, a specific growth rate of 0.30 h^{-1} corresponds to a specific substrate uptake rate of 0.42 g g^{-1} h^{-1}, whereas a specific uptake rate of 0.70 g g^{-1} h^{-1} corresponds to a specific growth rate of 0.51 h^{-1}. So, it is not clear wether the wash-out experiment was limited by the specific growth or by the specific substrate uptake rate. If the specific growth is the limiting rate ($\mu = 0.30\ h^{-1}$), then the specific uptake rate is adjusted at steady state by cell internal regulation in such a way that a maximum yield is achieved. After the dilution rate switch, the internal regulation is disturbed, and the higher uptake rates lead to an accumulation of metabolites. This would correspond with the measured data. On the other hand, if the specific uptake rate is the limiting rate ($r_{S\,max} = 0.70\ g\ g^{-1}\ h^{-1}$), then the specific growth rate is reduced after the switch, leading to lower yields and accumulation. This would also correspond with the measured data. In addition, a specific growth rate of 0.51 h^{-1} corresponds well with the estimated μ_{max} on phenol (0.48 h^{-1}). Because the same metabolic routes are involved during growth on phenol and benzoate, the second explanation seems more appropriate. However, no specific growth rates higher than 0.30 h^{-1} were measured experimentally for benzoate, Therefore the following calculation is based on the more conservative values.

Equation [4. 27], extended by the third substrate, leads to:

$D_{crit.\,(b)} = 0.92\ h^{-1}$

$D_{crit.\,(p)} = 1.08\ h^{-1}$

$D_{crit.\,(s)} = 2.67\ h^{-1}$

Therefore, benzoate is expected to accumulate first in the reactor, at a dilution rate three times higher than the critical dilution rate for this compound alone. The values for the other two substrates are not relevant, because accumulation, which takes place at dilution rates above the lowest $D_{crit.}$, is not considered in the equation. It should be mentioned, however, that considerable higher benzoate conversion rates were obtained from the batch experiments on the mixture of all substrates. Because the mechanism leading to these higher rates is unknown, they were not used here for calculation of the critical dilution rates, which is based solely on the data derived from single substrate experiments.

As a rough approximation for the dilution rates, at which the other two substrates will accumulate, a mathematical simulation was carried out using the MATLAB software. The following mass balances and kinetics were used:

Mass balances:	$dC_{S\,(p)}/dt = D\,(C_{S\,0\,(p)} - C_{S\,(p)}) - r_{S\,(p)}\,C_X$	phenol	[4. 31]
	$dC_{S\,(b)}/dt = D\,(C_{S\,0\,(b)} - C_{S\,(b)}) - r_{S\,(b)}\,C_X$	benzoate	[4. 32]
	$dC_{S\,(s)}/dt = D\,(C_{S\,0\,(s)} - C_{S\,(s)}) - r_{S\,(s)}\,C_X$	sodium acetate	[4. 33]
	$dC_X/dt = (\mu - D)\,C_X$	biomass	[4. 34]
Kinetics:	$r_{S\,(p)} = r_{Smax\,(p)}\,C_{S\,(p)}/(K_{S\,(p)} + C_{S\,(p)})$	phenol	[4. 35]
	$r_{S\,(b)} = r_{Smax\,(b)}\,C_{S\,(b)}/(K_{S\,(b)} + C_{S\,(b)})$	benzoate	[4. 36]
	$r_{S\,(s)} = r_{Smax\,(s)}\,C_{S\,(s)}/(K_{S\,(s)} + C_{S\,(s)})$	sodium acetate	[4. 37]
	$\mu = Y_{X/S\,(p)}\,r_{S\,(p)} + Y_{X/S\,(b)}\,r_{S\,(b)} + Y_{X/S\,(s)}\,r_{S\,(s)}$	biomass	[4. 38]

Fig. (4. 67) shows the simulation result. Benzoate appears first at the dilution rate already calculated. Phenol follows up closely at $D_{crit.} = 1.05\ h^{-1}$, and sodium acetate being the last at $D_{crit.} = 1.49\ h^{-1}$. It should be mentioned, however, that no inhibition was build into the model, which may alter appearance of sodium acetate considerably. It was shown with the second A-stat and the simulation, that the third substrate further extends the useful dilution rate range and that the anabolic flow is able to operate with three different substrates up to dilution rates of $0.69\ h^{-1}$.

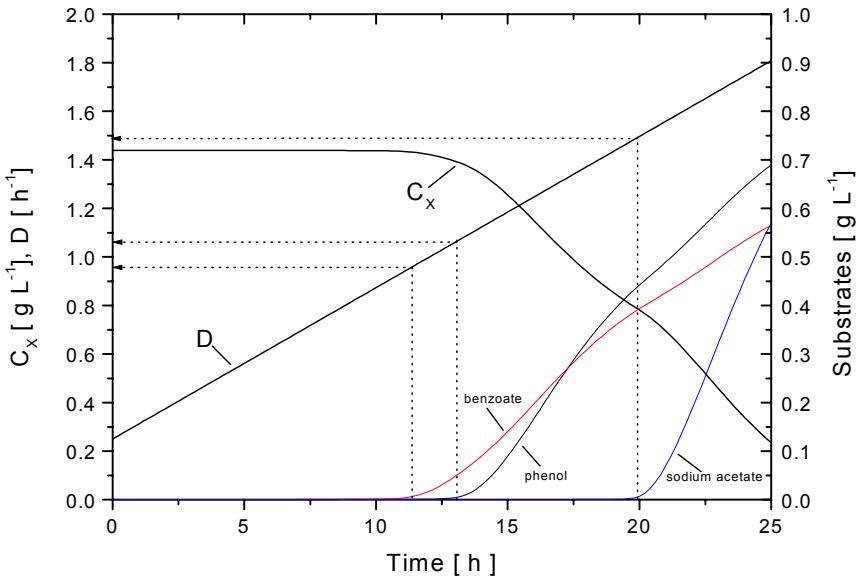

Fig. 4. 67. Simulation of the expected dilution rate to accumulate phenol, benzoate and sodium acetate in their mixture under the second A-stat conditions.

5. SUMMARY

Burkholderia cepacia G4 was cultivated in batch and continuous mode on the substrates phenol, benzoate, and sodium acetate applied as single substrates and in different substrate combinations.

Under steady state conditions in a chemostat, conversion of all three substrates to biomass and carbon dioxide was complete. This was shown for individual substrates as well as for substrate mixtures. In substrate mixtures, all compounds were mineralized simultaneously, no diauxic behaviour was observed. Phenol and benzoate are expected to be oxidized via the same *meta* cleavage pathway except for the first step leading to catechol. However, the molar biomass yields of phenol and benzoate are not identical, giving rise to the question if alternate pathways (i. e. *orho*-cleavage) might be induced in parallel. In the case of the benzoate, a green-yellow colouration of the culture suspension was observed, a clear indication of the formation of 2-hydroxymuconic semialdehyde (2-HMS), a pathway intermediate of the *meta*-route. Concentrations of 2-HMS, however, are low enough not to result in a deviation of the carbon recovery. For a substrate mixture composed of all three substrates, it was shown that the biomass can be calculated from the individual feed concentrations and the yield and maintenance coefficients derived from the steady states of the single substrate chemostats. Therefore, the culture behaves like the sum of individual cultivations.

Batch experiments were carried out to investigate the influence of higher substrate concentrations, and to detect possible inhibitions between the substrates. All batch experiments were performed as adjusted batches out of a chemostat to guarantee identical initial conditions if required.

All batch cultivations on phenol or benzoate, respectively, showed a decrease of the carbon recovery as long as substrate was present in the reactor, indicating formation of metabolites. After that, carbon recovery remained constant, so the accumulated metabolites resemble dead end metabolites. On sodium acetate, no deviation of the carbon recovery was observed. It was also demonstrated that after an up-shift of the substrate concentration (chemostat ⇒ batch), the cells show a step up of the specific rates immediately, and after that the specific rates are increasing slowly, indicating a slow adaptation to the changed conditions. The initial step up of the specific rates is dependent on the culture history or the growth rate of the preceding chemostat, respectively. This was shown for benzoate by mathematical simulation. For phenol, influence of culture history was already stated by Sokoł (1987). The following adaptation phase (increase of specific growth and substrate consumption rates) is too slow for enzyme regulation (milliseconds), but the time constant corresponds with genetic regulation. Therefore it is probable that the cells control their enzyme level, adjusting it to face the current conditions. This corresponds with the theory of Kovárová-Kovar and Egli (1998), who state that the cells constantly adapt to changing conditions by varying their stoichiometric and kinetic parameters.

It was also shown for phenol and benzoate, that the adaptation phase is accompanied by a decoupling of the catabolic and anabolic flows, most probably the reason for the accumulation of metabolites. Why the accumulated metabolites are not further metabolized after substrate depletion is unclear. Perhaps accumulation leads to chemical oxidation which prevents further enzymatic conversion. On sodium acetate, there was also a slow adaptation observed after a shift-up, but for this substrate no deviation of the carbon balance was detected. Therefore, no decoupling of catabolism and anabolism happens here. Acetate also shows no substrate inhibition. On benzoate, a slight substrate inhibition was detected, which is demonstrated by the time courses of OTR and CTR. Phenol faces a high substrate inhibition, which is already well known from the literature. Phenol also shows the most complex kinetic behaviour of the three substrates. The kinetic equation, derived from Schröder et al. (1997), from unstable steady states for the same strain, does not describe phenol conversion in batch culture. In addition to the influence of the culture history, the decoupling, and the adaptation phase, there is a further not yet known influence factor, leading to a slow phenol degradation in batch Sh-15 compared with Sh-14, despite of a higher growth rate of Sh-15 in the preceding chemostat. Both experiments were carried out using nearly identical initial concentrations in the batch.

For substrate mixtures of phenol and sodium acetate, a strong inhibition of phenol on acetate conversion was detected, reducing acetate conversion rates considerably compared with cultivation on sodium acetate alone. Acetate, on the other hand, reduced the phenol conversion slightly, therefore both substrates are cross inhibitory. For substrate mixtures of phenol and benzoate, no inhibition versus the other substrate was detected. For all substrate compositions, whether two or three substrates were used, degradation of the compounds was always simultaneously. The yellow colouration was detected in all cultivations where benzoate was present.

For substrate mixtures of all three compounds, a similar inhibitory pattern for the two-substrate cultivations was expected, but the experiments proved different. Although all compounds were metabolized simultaneously, benzoate was by far the fasted compound to be mineralized. In all three batches, benzoate was completely used up after less than 0.5 hours, considerably faster than in combination with phenol alone. However, the only exception was batch Sh-32 on phenol-benzoate, who showed a similar fast benzoate conversion than in the ternary mixtures. In this batch, the initial benzoate concentration was comparable to that in the ternary mixtures and lower than in the other batches on phenol-benzoate. Therefore, the low initial benzoate concentration might also be the cause for the rapid benzoate conversions in the ternary mixtures and batch Sh-32. Acetate, on the other hand, did not face the severe inhibition by phenol. In the presence of the third substrate benzoate, although benzoate was completely used up after 0.5 hours, acetate conversion rates are much faster than in corresponding phenol-sodium acetate cultivations.

A-stat cultivations and wash-out experiments were carried out to access the maximum performance of the cells. For benzoate, the maximum specific growth rate was estimated from a wash-out experiment to be 0.30 h^{-1}. This experiment was subject to metabolite formation. For sodium acetate, a wash-out experiment leads to a maximum growth rate of 0.60 h^{-1}, and for phenol, the maximum specific growth rate was estimated as 0.48 h^{-1}. An A-stat cultivation and a wash-out experiment lead to the same value. This value is considerably higher than the one published by Schröder et al. (1997), which was derived from unstable steady states on phenol (0.30 h^{-1}).

It seems that this strain has a complex regulation of its metabolic flows. In general, A-stat and chemostates resemble each other. Perhaps a controlled (unstable) steady state lead to small disturbances by the controls, which might lead to formation of regulatory compounds to reduce metabolic flows. A-stats are well suited to replace chemostats to get more information in shorter time. It was shown by a comparison that the use of acceleration factors up to 0.06 h^{-2} gave identical results as in a chemostat. It was also demonstrated by A-stat cultivations, that the presence of more than one substrate in the feed shifts the critical dilution rate, at which the first substrate will appear in the reactor, to higher values. With phenol, benzoate and sodium acetate in the feed, the strain was cultivated in an A-stat up to a dilution rate of 0.69 h^{-1} without accumulation of any of the substrates. With phenol alone, the maximum dilution rate in A-stat is only 0.48 h^{-1}. Therefore, additional substrates can expand the useful dilution rate range of a continuous cultivation considerably without sacrificing complete mineralization of all substrates present in the feed.

In this work, it was shown, that toxic phenol can be completely and simultaneously mineralized in the presence of alternating substrates, whether they are metabolized by the same catabolic route or by a complete different one. However, up-shifts of the substrate concentration(s) have to be avoided to prevent formation of metabolites. The complex regulation of the cell could not be addressed in detail in this work. Herefore, pathway intermediates of the different involved pathways in the cell have to be measured to try identify regulatory compounds. The theory of variable yield and maintenance coefficients, however, was proven for this biological system. The complex regulatory network of the cells, which is influenced by the culture history, the type of substrate, and the process dynamics leads to different parameter values according to the cultivation conditions.

6. ZUSAMMENFASSUNG

In dieser Arbeit wurde das Abbauverhalten einer Reinkultur von *Burkholderia cepacia* G4 hinsichtlich der Verbindungen Phenol, Benzoat und Acetat untersucht. Die drei Verbindungen stellten dabei die alleinigen Kohlenstoff- und Energiequellen für den Mikroorganismus dar. Untersucht wurde der Abbau der Einzelsubstanzen und von Gemischen derselben bei unterschiedlichen Kultivierungsbedingungen (Satzbetrieb, kontinuierlich, stationär, dynamisch).

Unter stationären Bedingungen bei Substratlimitierung in Chemostat wurde für alle drei Verbindungen ein vollständiger Umsatz zu Biomasse und Kohlendioxid beobachtet, sowohl bei den Einzelsubstanzen wie auch bei den Substratgemischen. Es erfolgte ein simultaner Abbau aller Substrate in den Gemischen, eine Diauxie trat nicht auf. Für Phenol und Benzoat kann mit Ausnahme des ersten Schrittes zum Brenzcatechin der gleiche Abbauwege (*meta*-Spaltung) angenommen werden. Die experimentellen Ergebnisse zeigten jedoch unterschiedliche molare Biomasseausbeutekoeffizienten, ein Hinweis darauf, dass möglicherweise auch alternative Stoffwechselwege (z. B. *ortho*-Spaltung) parallelinduziert werden. Beim Benzoatabbau wurde eine Gelbgrünfärbung beobachtet, die auf 2-Hydroxymuconat-semialdehyd (2-HMS) hinweist, eine Verbindung, die charakteristisch für den *meta*-Abbauwege ist. Die Messung der Konzentrationen des 2-HMS zeigte, dass diese Verbindung nicht für das große Kohlenstoffdefizit verantwortlich ist, das bei Versuchen unter dynamischen Bedingungen beim Benzoatabbau gefunden wurde. Für stationäre Bedingungen im Chemostat konnte weiterhin gezeigt werden, dass sich die Biomasse additiv aus den Einzelsubstraten zusammensetzt, d. h. die stöchiometrischen Daten aus den Experimenten mit den Einzelsubstraten können auf das Gemisch übertragen werden. Die Abbauleistung bei einem Substratgemisch läßt sich damit aus den Daten der Einzelsubstrate vorausberechnen.

Zur Untersuchung des Abbauverhaltens unter höheren Substratkonzentrationen (Substratüberschuß) und um mögliche Inhibitionen der Substrate untereinander oder gegen sich selbst zu erkennen, wurden Satzversuche durchgeführt. Zur möglichst guten Vergleichbarkeit wurden alle Versuche aus einem Chemostat heraus als “Adjusted batch” durchgeführt, d. h. die Ausgangsbedingungen eines Satzversuches können gezielt eingestellt und bei Bedarf exakt wiederholt werden.

In Gegensatz zu den stationären Bedingungen im Chemostat tritt bei allen Satzversuchen mit Phenol oder Benzoat ein Kohlenstoff-Bilanzdefizit auf, dass solange zunimmt bis das Substrat im Reaktor vollständig umgesetzt ist. Es kann also die Bildung und Akkumulation von Stoffwechselprodukten angenommen werden. Nach dem vollständigen Substratumsatz bleibt das Bilanzdefizit konstant, d. h. die gebildeten Stoffwechselprodukte verhalten sich wie Dead-End-Metabolite.

Beim Acetatumsatz (als alleinige C-Quelle) wurde keine Abweichung der Kohlenstoffbilanz beobachtet. Es wurde ferner beobachtet, dass nach einem Substratsprung zu höheren Konzentrationen (Chemostat ⇒ Satzversuch) eine sofortige Steigerung der spezifischen Umsatzrate und der spezifischen Wachstumsrate stattfindet, die aber noch nicht ihren Maximalwerten entspricht. Danach steigern sich die spezifischen Raten langsam weiter, was auf eine langsame Adaptation an die geänderten Umgebungsbedingungen hinweist. Die Größe des Initialen Sprungs der spezifischen Raten ist abhängig von der Durchflußrate des Ausgangschemostaten ("Kulturhistorie"). Dies konnte für Benzoat durch mathematische Modellierung gezeigt werden. Für Phenol wurde der Einfluß der Kulturhistorie schon 1987 von Sokoł festgestellt. Die folgende Adaptationsphase (Steigerung der Wachstums- und Substratabbaugeschwindigkeiten) ist zu langsam für eine Enzymregulation (schnelle Zeitkonstante, Millisekunden), korreliert aber mit Zeitkonstanten die typisch für eine genetische Regulation sind (Minuten bis Stunden). Es ist daher wahrscheinlich, dass die Zelle ihre Enzymkonzentration reguliert und den jeweiligen Umgebungsbedingungen anpaßt. Dies stimmt mit der Hypothese von Kovàrovà-Kovar und Egli (1998) überein, die postulierten, dass sich die Zellen ständig an wechselnde Umgebungsbedingungen durch Variation ihrer kinetischen und stöchiometrischen Parameter anpassen.

Für Phenol und Benzoat konnte gezeigt werden, dass während der Adaptationsphase eine Entkopplung des katabolischen und des anabolischen Flusses eintritt, die höchstwahrscheinlich für die Metabolitakkumulation verantwortlich ist. Es ist jedoch nicht klar, warum die akkumulierten Metabolite nach dem vollständigen Substratumsatz nicht weiter abgebaut werden. Möglicherweise verhindert eine chemische Oxidation einen weiteren Umsatz. Für Acetat wurde ebenfalls eine langsame Adaptation nach einer Änderung der Wachstumsbedingungen beobachtet, aber es tritt kein Kohlenstoffbilanzdefizit auf. Eine metabolische Entkopplung spielt daher beim Acetatabbau keine Rolle.

Beim Abbau von Acetat konnte keine Substratinhibition festgestellt werden. Benzoat zeigt dagegen eine schwache Substratinhibition, was auch an den zeitlichen Verläufen der Sauerstoffaufnahmerate OTR und der Kohlendioxidbildungsrate CTR ersichtlich ist. Phenol inhibiert seinen eigenen Abbau (stärkere Substratinhibition) bereits in relativ kleinen Konzentrationen, was schon aus den Literatur bekannt ist. Phenol zeigt auch das komplexeste kinetische Verhalten der drei untersuchten Substrate. Die kinetische Gleichung, die Schröder 1997 für den selben Stamm aus geregelten instabilen stationären Zuständen abgeleitet hat, kann das Abbauverhalten im Satzversuch nicht beschreiben. Die Kinetik, die stationäre Zustände beschreiben kann, läßt sich somit nicht auf dynamische Bedingungen übertragen. Zusätzlich zum Einfluß der Kulturhistorie, der metabolischen Entkopplung und der Adaptationsphase gibt es noch eine weitere bis jetzt noch nicht identifizierte Einflußgröße auf die Kinetik, die zu einem langsameren Phenolabbau bei dem Versuch Sh-15 gegenüber dem Versuch Sh-14 führte, obwohl die Durchflußrate des vorausgehenden Chemostaten beim Versuch Sh-15 größer war. Beide Experimente wurden mit identischen Substratausgangskonzentrationen durchgeführt.

Bei Substratgemischen aus Phenol und Natriumacetat wurde eine starke Inhibition des Acetatabbaus durch Phenol festgestellt, die zu einer deutlich geringeren Acetatabbaurate beim Gemisch verglichen mit dem Abbau von Acetat als alleinige Kohlenstoffquelle führt. Auf der anderen Seite wurde auch eine leichte Inhibition des Phenolumsatzes durch Acetat entdeckt, so dass sich beide Substrate gegenseitig inhibieren. Demgegenüber konnte bei Gemischen aus Phenol und Benzoat keine gegenseitige Inhibition festgestellt werden. Bei allen Substratgemischen, unabhängig davon ob sie aus zwei oder drei Verbindungen bestanden, erfolgte der Abbau stets simultan. Bei allen Versuchen, an denen Benzoat beteiligt war, wurde immer die charakteristische Gelbfärbung von 2-HMS beobachtet.

Für Substratgemische aus 3 Komponenten wurde ein abweichendes Abbauverhalten gegenüber Mischungen aus zwei Komponenten erhalten. Obwohl auch hier ein simultaner Abbau aller drei Substrate beobachtet wurde, ist Benzoat in allen Fällen das bevorzugte Substrat mit dem schnellsten Umsatz. In allen drei Satzversuchen mit dem Dreikomponentengemisch war Benzoat noch weniger als 0,5 Stunden vollständig umgesetzt, deutlich schneller als mit Phenol allein. Die einzige Ausnahme war der Satzversuch Sh-32 auf Phenol und Benzoat, der einen vergleichbar schnellen Benzoatumsatz wie in den ternären Gemischen zeigte. Bei diesem Versuch war die Benzoatanfangskonzentration vergleichbar groß wie bei den ternären Gemischen aber geringer als bei den Gemischen mit Phenol allein. Möglicherweise könnte also die niedrige Anfangskonzentration von Benzoat der Grund für die hohen Benzoatumsatzraten sein, obwohl Benzoat nur eine geringe Substratinhibition zeigt. Die ausgeprägte Inhibition des Acetatumsatzes durch Phenol ist bei den ternären Gemischen nicht erkennbar. Der einzige Unterschied zum Phenol-Acetat-Gemisch ist die Anwesenheit von Benzoat. In Gegenwart von Benzoat sind die Acetatabbauraten deutlich höher, und das auch nachdem Benzoat nach 0,5 Stunden bereits vollständig umgesetzt war.

Um die maximale Wachstumsgeschwindigkeit und Abbauleistung zu ermitteln wurden A-stat und Auswaschversuche durchgeführt. Auf Benzoat wurde eine maximale spezifischen Wachstumsgeschwindigkeit von 0,30 h^{-1} in einem Auswaschversuch ermittelt. Bei diesem Versuch trat jedoch Metabolitakkumulation auf. Ebenfalls aus Auswaschversuchen wurden die maximalen spezifischen Wachstumsraten von Acetat und Phenol zu 0,60 h^{-1} und 0,48 h^{-1} ermittelt. Für Phenol wurden identische maximale Wachstumsraten aus einem A-stat-Versuch und einem Auswaschversuch erhalten. Dieser Wert ist deutlich größer als der den Schröder 1997 aus geregelten instabilen stationären Zuständen für den gleichen Stamm und das Substrat Phenol ermittelt hat (0,30 h^{-1}).

Offensichtlich sind die metabolischen Flüsse bei diesem Stamm komplex reguliert. Ein A-stat-Versuch erzeugt quasi stationäre Bedingungen, so dass sich A-stat und Chemostat in dieser Hinsicht entsprechen. Möglicherweise führen kleine Regelungabweichungen bei geregelten instabilen stationären Zuständen zu Störungen der metabolischen Flüsse, die dann über zellinterne Regelvorgänge zu einer Reduzierung der metabolischen Flüsse, und damit zu einem früheren Auswaschen führen.

A-stat-Kultivierungen sind generell gut geeignet um Chemostate zu ersetzen und mehr Informationen in kürzerer Zeit zu erhalten. Es konnte durch einen Vergleich gezeigt werden, dass A-stat-Versuche mit Beschleunigungsfaktoren bis zu 0,06 h^{-2} identische Resultate mit Chemostatversuchen ergeben. Es konnte auch durch A-stat-Versuche gezeigt werden, dass sich bei Substratgemischen die kritische Durchflußrate, bei der das erste Substrat im Reaktor akkumuliert, zu höheren Werten verschiebt. Mit dem ternären Substratgemisch konnte in einem A-stat die Durchflußrate bis 0,69 h^{-1} gesteigert werden ohne dass eines der drei Substrate akkumuliert. Mit Phenol als einziger Kohlenstoffquelle betrug die maximale Durchflußrate im A-stat 0,48 h^{-1}. Die Anwesenheit weiterer Substrate zusätzlich zu einer toxischen Zielkomponente können also die Reaktorleistung bei vollständiger Mineralisierung aller Kohlenstoffquellen erhöhen.

In dieser Arbeit konnte gezeigt werden dass die toxische Verbindung Phenol vollständig und simultan in Gegenwart weiterer Kohlenstoffquellen abgebaut werden kann, unabhängig davon ob die anderen Verbindungen über den gleichen oder andere Abbauwege mineralisiert werden. Substratsprünge sollten jedoch möglichst vermieden werden um eine Metabolitakkumulation zu verhindern. Die komplexe Regulation der Zellen konnte im Detail in dieser Arbeit nicht aufgeklärt werden. Dazu müssen einzelne Abbauwegintermediate gemessen werden um regulatorisch wirkende Verbindungen zu erkennen. Dennoch konnte die Theorie variabler stöchiometrischer und kinetischer Parameter für dieses biologische System bewiesen werden. Das komplexe regulatorische Netzwerk der Zellen, das durch die Kulturhistorie, das Substrat und die Prozessdynamik beeinflußt wird, führt zu unterschiedlichen Parameterwerten je nach den Kulturbedingungen.

7. REFERENCES

Allsop, P. J., Chisit, Y., Moo-Young, M. and Sullivan, G. R. (1993). Dynamics of phenol degradation by *Pseudomonas putida*. Biotechnol. Bioeng. **41**: 572-580.

Ampe, F. and Lindley, N. D. (1995). Acetate utilization is inhibited by benzoate in *Alcaligenes eutrophus*: Evidence for transcriptional control of the expression of acoE coding for acetyle coenzyme A synthetase. J. Bacteriol. **177**: 5826-5833.

Ampe, F. and Lindley, N. D. (1996). Flux limitations in the *ortho* pathway of benzoate degradation of *Alcaligenes eutrophus*: metabolite overflow and induction of the *meta* pathway at high substrate concentrations. Microbiol. **142**: 1807-1817.

Andrews, J. F. (1968). A mathematical model for the continious culture of microorganisms utilizing inhibitory substrates. Biotechnol. Bioeng. **10**: 707-723.

Anselmo, A. M., Mateus, M., Cabral, J. M. S. and Novais, J. M. (1985). Degradation of phenol by immobilized cells of *Fusarium flocciferum*. Biotechnol. Lett. **7** (12): 889-894.

Anselmo, A. M. and Novais, J. M. (1992). Biological treatment of phenolic wastes: comparison between free and immobilized cell systems. Biotechnol. Lett. **14**: 239-244.

Bauer, J. E. and Capone, D. G. (1985). Effects of four aromatic pollutants on microbial glucose metabolism and thymidine incorporation in marine sediments. Appl. Environ. Microbiol. **49**: 828-835.

Bettmann, H. and Rehm, H. J. (1984). Degradation of phenol by polymer entrapped microorganisms. App. Microbiol. Biotechnol. **20**: 285-290.

Bielefeldt, A. R. and Stensel, H. D. (1999). Biodegradation of aromatic compounds and TCE by a filamentous bacteria-domenated consortium. Biodegradation, **10**: 1-13.

Bitton, G. and Koopman, B. (1986). Biochemical tests for toxicity screening: toxicity testing microorganisms. G. Bitton and B. J. Dutka, Eds. CRC press, Boca Raton, FL, pp. 27-55.

Bouwer, E. J. and Zehnder, A. J. B. (1993). Bioremediation of organic compounds putting microbial metabolism to work. Trends Biotechnol. **11**: 360-367.

Bremer, P. (1994). Die Kinetik des Phenolabbaus in einem geschlossen System mit einer Reinkultur von *Pseudomonas cepacia* G4. Diploma-work, Bayrische Julius-Maximilians-Universität, Würzburg, Germany.

Brilon, C., Beckmann, W. and Knackmuss, H. J. (1981). Catabolism of naphthalenesulfonic acids by *Pseudomonas sp*. A3 and *Pseudomonas sp*. C22. Appl. Environ. Microbiol. **42**: 44-55.

Brinkmann, U. and Babel, W. (1992). Simultaneous utilization of heterotrophic substrates by *Hansenula polymorpha* MH30 results in enhanced growth rates. Appl. Microbiol. Biotechnol. **37**: 98-103.

Buchtmann, C., Kies, U., Deckwer, W.-D. and Hecht, V. (1997). Performance of three phase fluidized bed bioreactor for quinoline degradation on various supports at steady state and dynamic conditions. Biotechnol. Bioeng. **56**: 295-303.

Buswell, J. A. (1975). Metabolism of phenol and cresols by *Bacillus stearothermophilus*. J. Bacteriol. **124**: 1077-1083.

Cain, R. B., Bilton, R. F. and Darrah, J. A. (1968). The metabolism of aromatic acids by micro-organisms: metabolic pathways in the fungi. Biochem. J. **108**: 797-828.

Cain, R. B. and Farr, D. R. (1968). Metabolism of arylsulphonates by microorganisms. Biochem. J. **106**: 859-877.

Caldwell, M. E. and Suflita, J. M. (2000). Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. Environ. Sci. Technol. **34**: 1216-1220.

Chi, C. T. and Howell, J. A. (1976). Transient behavior of a continuous stirred tank biological reactor utilizing phenol as an inhibitory substrate. Biotechnol. Bioeng. **18**: 63-80.

Cho, Y-G., Rhee, S-K. and Lee, S-T. (2000). Influence of phenol on biodegradation of p-nitrophenol by freely suspended and immobilized *Nocardioides sp*. NSP41. Biodegrad. **11**: 21-28.

Commandeur, L. C. M. and Parsons, J. R. (1990). Degradation of halogenated aromatic compounds. Biodegradation, **1**: 207-220.

Cozzone, A. J. (1998). Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. Ann. Rev. Microbiol. **52**: 127-164.

Davies, J. I. and Evans, W. C. (1964). Oxidative metabolism of naphthalene by soil Pseudomonads. Biochem. J. **91**: 251-261.

D' Adamo, P. D., Rozich, A. F. and Gaudy Jr., A. F. (1984). Analysis of growth data with inhibitory carbon sources. Biotechnol. Bioeng. **26**: 397-402

De-Lipthay, J. R., Barkay, T., Vekova, J. and Sϕrenson, S. J. (1999). Utilization of phenoxyacetic acid, by strains using either the *ortho* or *meta* cleavage of catechol during phenol degradation, after conjugal transfer of tfdA, the gene encoding a 2,4-dichlorophenoxyacetic acid/2-oxoglutarate dioxygenase. Appl. Microbiol. Biotechnol. **51**: 207-214.

Dikshitulu, S., Baltzis, B. C., Lewandowski, G. A. and Pavlou, S. (1993). Compitition between two microbial populations in a sequencing fed-batch reactor: theory, experimental verification, and implications for waste treatment applications. Biotechnol. Bioeng. **42**: 643-656.

Duboc, P. (1997). Transient growth of Saccharomyces cervisiae, a quantitative approach. Ph. D. thesis, Swiss Federal Institute of Technology, Lausanne, Switzerland.

Duboc, P., von Stockar, U. and Villadsen, J. (1998). Simple generic model for dynamic experiments with *Saccharomyces cervisiae* in continuous culture: Decoupling between anabolism and catabolism. Biotech. Bioeng. **60**: 180-189.

Edwards, V. H. (1970). The influence of high substrate concentrations on microbial kinetics. Biotechnol. Bioeng. **12**: 679-712.

Edwards, V. H., Ko, C. R. and Balogh, A. (1972). Dynamics and control of continuous microbial propagators subject substrate inhibition. Biotechnol. Bioeng. **14**: 939-974.

Egli, T. (1995). The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. Adv. Microb. Ecol. **14**: 305-386.

Esener, A. A., Roels, J. A., Kossen, N. W. F. and Roozenburg, J. W. H. (1981). Description of microbial growth behaviour during the wash-out phase; determination of the maximum specific growth rate. European J. Appl. Microbiol. Biotechnol. **13**: 141-144.

Farrell, A. and Quilty, B. (1999). Degradation of mono-chlorophenols by a mixed microbial community via a *meta*-cleavage pathway. Biodegradation, **10**: 353-362.

Fava, F., Armenante, P. M., Kafkewitz, D. and Marchetti, L. (1995). Influence of organic and inorganic growth supplements on the aerobic biodegradation of chlorobenzoic acid. Appl. Microbiol. Biotechnol. **43**: 171-177.

Feist, C. and Hegeman, G. D. (1969a). Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. J. Bacteriol. **100**: 869-877.

Feist, C. and Hegeman, G. D. (1969b). Regulation of the *meta* cleavage pathway for benzoate oxidation by *Pseudomonas putida*. J. Bacteriol. **100**:1121-1123.

Fischer, P. (1989). Reaktionskinetische Untersuchungen zum mikrobiellen Phenolabbau. TU Berlin, Fachbereich Lebensmittel- und Biotechnologie, Dissertation.

Folsom, B. R. and Chapman, P. J. (1991). Performance characterization of a model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia* G4. App. Environment. Microbiol. **57**: 1602-1608.

Folsom, B. R., Chapman, P. J. and Pritchard, P. H. (1990). Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: Kinetics and interactions between substrates. Appl. Environment. Microbiol. **56**: 1279-1285.

Gaal, A. and Neujahr, H. Y. (1979). Metabolism of phenol and resorcinol in *Trichosporon cutaneum*. J. Bacteriol. **137**: 13-21.

Gaal, A. and Neujahr, H. Y. (1980). Cis,cis-muconate cyclase from *Trichosporon cutaneum*. Biochem. J. **191**: 37-43.

Ghadi, S. and Sangodkar, U. M. X. (1994). Identification of a *meta*-cleavage pathway for metabolism of phenoxyacetic acid and phenol in *Pseudomonas cepacia* AC1100.Biochem. Biophysical Research Communications, **204**: 983-993.

Gerritse, J., Schut, F. and Gottschal, J. C. (1992). Modelling of mixed chemostat cultures of an aerobic bacterium, *Comamonas testosteroni*, and an anaerobic bacterium, *Veillonella alcalescens*: Comparison with experimental data. Appl. Environ. Microbiol. **58**: 1466-1476.

Gibson, D. T. (1971). The microbial oxidation of aromatic compounds. Crit. Rev. Microbiol. **1**: 199-223.

Goudar, C. T., Ganji, S. H., Pujar, B. G. and Strevett, K. A. (2000). Substrate inhibition kinetics of phenol biodegradation. Water Environment Research, **72**: 50-55.

Götz, P. and Reuss, M. (1997). Dynamics of microbial growth: modelling time delays by introducing a polymerization reaction. J. Biotechnol. **58**: 101-114.

Grady, C. P. L. (1985). Biodegradation: its measurement and microbiological basis. Biotechnol. Bioeng. **27**: 660-674.

Grady, C. P. L., Smtes, B. F. and Barbeau, D. S. (1996). Variability in kinetic parameter estimates: a review of possible causes and proposed terminology. Water Res. **30**: 742-748.

Groenewegen, P. E. J., Breeuwer, P., van Helvoort, J. M. L. M., Langenhoff, A. A. M., de Vries, F. P. and de Bont, J. A. M. (1992). Novel degradative pathway of 4-nitrobenzoate in *Comomonas acidovorans* NBA-10. J. General Microbiol. **138**: 1599-1605.

Haigler, B. E., Pettigrew C. A. and Spain, J. C. (1992). Biodegradation of mixtures of substituted benzens by *Pseudomonas sp*. strain JS150. Appl. Environ. Microbiol. **58**: 2237-2244.

Haldane, J. B. S. (1930). Enzymes. Longmans, Londen.

Hamzah, R. Y. and Al-Baharna, B. S. (1994). Catechol ring cleavage in *Pseudomonas cepacia*: the simultaneous induction of *ortho* and *meta* pathways. Appl. Microbiol. Biotechnol. **41**: 250-256.

Harayama, S., Mermod, N., Rekik, M., Lehrbach, P. R. and Timmis, K. N. (1987). Roles of the divergent branches of the *meta*-cleavage pathway in the degreadation of benzoate and substituted benzoates. J. Bacteriol. **169**: 558-564.

Hecht, V., Brebbermann, D., Bremer, P. and Deckwer, W.-D. (1994). Cometabolic degradation of trichloroethylene in a bubble column bioscruber. Biotechnol. Bioeng. **47**: 461-469.

Hecht, V., Langer, O. and Deckwer, W.-D. (2000). Degradation of phenol and benzoic acid in a three-phase fluidized-bed reactor. Biotechnol. Bioeng. **70**: 391-399.

Herbert, D., Elsworth, R. and Telling, R. C. (1956). The continuous culture of bacteria: a theoretical and experimental study. J. Gen. Microbiol. **14**: 601-622.

Hernandez, E. and Johnson, M. (1967). Energy supply and cell yield in aerobically grown microorganisms. J. Bacteriol. **94**: 996-1001.

Hickey, W. J. and Focht, D. D. (1990). Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. Appl. Environ. Microbiol. **56**: 3842-3850.

Hill, G. A. and Robinson, C. W. (1975). Substrate inhibition kinetics: phenol degradation by *pseudomonas putida*. Biotechnol. Bioeng. **17**: 1599-1615.

Hinteregger, C., Leitner, R., Loidl, M., Ferschl, A. and Streichsbier, F. (1992). Degradation of phenol and phenolic compounds by *Pseudomonas putida* EKII. Appl. Microbiol. Biotechnol. **37**: 252-259.

Hofmann, K. H. (1986). Oxidation of naphthalene by *Saccharomyces cervisiae* and *Candida utilis*. J. Basic Microbiol. **26**: 109-111.

Hofmann, K. H. and Kruger, A. (1985). Induction and inactivation of phenol hydroxylase and catechol oxygenase in *Candida maltosa* L4 in dependence on the carbon source. J. Basic Microbiol. **25**: 373-379.

Holms, W. H. (1987). Control of flux through the citric acid cycle and the glyoxylate bypass in *Escherichia coli*. Biochem. Soc. Symp. **54**: 17-31.

Hoyt, J. C., Robertson, E. F., Berlyn, K. A. and Reeves, H. (1988). *Escherichia coli* isolate lyase: properties and comparisons. Biochimica et Biophysica Acta, **966**: 30-35.

Hughes, E. J. and Bayly, R. C. (1983). Control of catechol *meta* cleavage pathway in *Ralstonia eutropha*. J. Bacteriol. **154**: 1363-1370.

Hughes, E. J., Bayly, R. C. and Skurray, R. A. (1984). Evidence for isofunctional enzymes in the degradation of phenol, m-and p-toluate, and p-cresol via catechol *meta* cleavage pathways in *Alcaligenes eutrophus*. J. Bacteriol. **158**: 79-83.

Hutchinson, D. H. and Robinson, C. W. (1988). Kinetics of the simultaneous batch degradation of p-cresol and phenol by *pseudomonas putida*. Appl. Microbiol. Biotechnol. **29**: 599-604.

Johnson, B. F. and Stanier, R. Y. (1971). Dissimilation of aromatic compounds by *Alcaligenes eutrophus*. J. Bacteriol. **107**: 468-475.

Katayama-Hirayama, K., Tobita, S. and Hirayama, K. (1991). Metabolic pathway of phenol in *Rhodotorula rubra*. J. Gen. Appl. Microbiol. **37**: 379-388.

Kehlenbeck, M. (1998). Untersuchungen zum Mikrobiellen Phenolabbau durch *Burkholderia cepacia* G4 in Gegenwart von Glucose. Diploma work, TU. Braunschweig, Germany.

Keweloh, H., Heipieper, H. J. and Rehm, H. J. (1989). Tolerance of bacteria against phenol after immobilization in Ca-alginate. Dechema-Biotechnol. Conferences, **3**: 797-800.

Keweloh, H., Weyrauch, G. and Rehm, H.-J. (1990). Phenol induced membrane changes in free and immobilized *Escherichia coli*. Appl. Microbiol. Biotechnol. **33**: 66-71.

Kies, U. (1998). Modellierung des Abbaus von Chinolin durch *Comamonas acidovorans*. PhD-work, TU. Braunschweig, Germany.

Kilpi, S., Backstrom, V. and Korhola, M. (1983). Degradation of catechol by *Pseudomonas sp.* HV3. FEMS Microbiol. Lett. **18**: 1-15.

Klečka, G. M. and Maier, W. J. (1988). Kinetics of microbial growth on mixtures of pentachlorophenol and chloronated aromatic compounds. Biotechnol. Bioeng. **31**: 28-335.

Knackmuss, H. and Helweg, M. (1978). Utilization and cooxidation of chlorinated phenols by *Pseudomonas sp.* B13. Arch. Microbiol. **117**: 1-7.

Kornberg, H. L. and Madsen, N. B. (1957). Synthesis of C_4- dicarboxylic acids from acetate by a "glyoxylate bypass"of the tricarboxylic acid cycle. Prelimenary Notes, **24**: 651-653.

Kotturi, G., Robinson, C. W. and Inniss, W. E. (1991). Phenol degradation by a psychrotrophic strain of *Pseudomonas putida*. Appl. Microbiol. Biotechnol. **34**: 539-543.

Kovárová-Kovar, K., and Egli, T. (1998). Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbiol. Molecul. Biol. Rev. **62**: 646-666.

Krug, M. and Straube, G. (1986). Degradation of phenolic compounds by the yeast *Candida tropicalis* HP15. II. Some properties of the first two enzymes of the degradation pathway. J. Basic. microbiol. **26**: 271-281.

Kukor, J. J. and Olsen, R. H. (1991). Genetic organization and regulation of a *meta* cleavage pathway for catechols produced from catabolism of toluene, benzene, phenol, and cresols by *Pseudomonas pickettii* PKO1. J. Bacteriol. **173**: 4587-4594.

LaPat-Polasko, L. T., McCarty, P. L. and Zehnder, A. J. (1984). Secondary substrate utilization of methylene chloride by an isolated strain of *Pseudomonas sp.* Appl. Environ. Microbiol. **47**: 825-830.

Lendenmann, U. (1994). Growth kinetics of *Escherichia coli* with mixtures of sugars, PhD-work, Swiss Federal Institute of Technology, Switzerland.

Lendenmann, U. and Egli, T. (1998). Kinetics models for the growth of *Escherichia coli* with mixtures of sugars under carbon-limited conditions. Biotech. Bioeng. **59**: 99-107.

Léonard, D., Ben Youssef, C., Destruhaut, C., Lindley, N. D. and Queinnec, I. (1999). Phenol degradation by *Ralstonia eutropha*: Colorimetric determination of 2-hydroxymuconate semialdehyde accumulation to control feed strategy in fed-batch fermintations. Biotechnol. Bioeng. **65**: 407-415.

Léonard, D. and Lindley, N. D. (1998). Carbon and energy flux constraints in continuous cultures of *Alcaligenes eutrophus* grown on phenol. Microbio. **144**: 241-248.

Li, J-K. and Humphrey, A. E. (1989). Kinetic and fluorometric behavior of a phenol fermentation. Biotechnol. Lett. **11**: 177-182.

Livingston, A. G. (1991). Biodegradation of 3,4-dichloroanaline in a fluidized bed bioreactor and a steady state biofilm kinetic model. Biotechnol. Bioeng. **38**: 260-272.

Loh, K.-C. and Chua, S.-S. (2002). *Ortho* pathway of benzoate degradation in *Pseudomonas putida*: induction of *meta* pathway at high substrate concentrations. Enzym. Microb. Technol. **30**: 620-626.

Loh, K. C. and Wang, S. J. (1998). Enhancement of biodegradation of phenol and a non-growth substrate 4-chlorophenol by medium augmentation with conventional carbon sources. Biodegradation, **8**: 329-338.

Luong, J. H. T. (1987). Generalization of Monod kinetics for analysis of growth data with substrate inhibition. Biotechnol. Bioeng. **29**: 242-248.

Marison, L. W. (1988). Enzyme kinetics: biotechnology for engineers; biological systems in technological processes. A. Scrag, Ed. Ellis Harwood, Chichester, U. K., pp. 96-119.

Marita, R. Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. J. Can. Microbiol. **43**: 436-441.

McMullan, G. and Quinn, J. P. (1994). In nitro characterization of a phosphate starvation-independent carbon phosphorus bond cleavage activity in *Pseudomonas fluorescences* 23F. J. Bacteriol. **176** : 320-324.

Monod, J. (1942). Recherches sur la croissance des cultures bactériennes. Hermann et Cie, Paris, France.

Monod, J. (1949). The growth of bacterial cultures. Ann. Rev. Microbiol. **3**: 371-394.

Mörsen, A. and Rehm, H. J. (1990). Degradation of phenol by a defined mixed culture immobilized by adsorption on activated carbon and sintered glass. Appl. Microbiol. Biotechnol. **33**: 206-212.

Müller, C. (1994). Entwicklung einer Regelungsstrategie zur Erfassung von instabilen stationären Zuständen bei substratinhibiierten Systemen am Beispiel des Phenolabbaus durch *Pseudomonas cepacia* G4. Diplomarbeit, TU. Braunschweig.

Müller, R. H. and Babel, W. (1995). Determination of the Ks values during the growth of *Alcaligenes eutrophus* on phenol , 2,4-Dichlorophenoxyacetic acid and fructose. Acta Biotechnol. **15**: 347-353.

Müller, R. H., Bley, T. and Babel, W. (1995). Transient state cultivation as a means for determining maximum growth rates of microorganism in inhibition kinetics. J. Microbiol. Meth. **22**: 209-219.

Müller, R.H., Deckwer, W.-D. and Hecht, V. (1996). Degradation of chloro-and methylsubstituted benzoic acids by a genetically modified microorganism. Biotech. Bioeng. **51**: 528-537.

MüncnerovÁ, D. and Augustin, J. (1994). The influence of pH on growth kinetics of yeasts in the presence of benzoate as a sole carbon source. Folia Microbiol. **39**: 265-268.

Nakazawa, T. and Yokota, T. (1973). Benzoate metabolism in *Pseudomonas putida(arvilla)* mt-2: demonstration of two benzoate pathways. J. Bacteriol. **115**: 262-267.

Nelson, M. J. K., Montgomery, S. O., Mahaffey, W. R. and Pritchard, P. H. (1987). Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environment. Microbiol. **53**: 949-954.

Nelson, M. J. K., Montgomery, S. O., O'neill, E. J. and Pritchard, P. H. (1986). Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environment. Microbiol. **52**: 383-384.

Neujahr, H. Y. and Gaal, A. (1973). Phenol hydrolase from yeast. Purification and properties of the enzyme from *Trichosporon cutaneum*. Eur. J. Biochem. **35**: 386-400.

Neujahr, H. Y., Lindsjö, S. and Varga, J. M. (1974). Oxidation of phenol by cells and cell-free enzymes from *Candida tropicalis*. Antonie van Leeuwenhoek, **40**: 209-216.

Norbad, A., Hewlins, M. J. andCallely, A. G. (1989). 13C-NMR sudies of acetate and methanol metabolism by methylotrophic *Pseudomonas* strains. J. Gen. Microbiol. **135**: 1469-1477.

Noronha, S. B., Yeh, H. J. C., Spande, T. F. and Shiloach, J. (2000). Investigation of the TCA cycle and the Glyoxylate shnut in *Esherichia coli* BL21 and JM109 using ^{13}C-NMR/MS. Biotech. Bioeng. **68**: 316-327.

Nozaki, M., Kagamiyama, H. and Hayaishi, O. (1963). Crysalization and some properties of metapyrocatechase. Biochem. Biophys. Res. Commun. **11**: 65-69.

Ornston, L. N. (1966). The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. J. Biol. Chem. **241**: 3800-3810.

Ornston, L. N. and Yeh, W-K. (1982). Reccuring themes and repeated sequences in metabolic evolution: biodegradation and detoxification of environmental pollutants. CRC press, Boca Raton, Fla. p. 105-126.

Ottow, J. C. G. and Zolg, W. (1974). Improved procedure and colorimetric test for the detection of *ortho* and *meta*-cleavage of protocatechuate by *Pseudomonas* isolates. Can. J. Microbiol. **20**: 1059-1061.

Oude Elfernik, S. T. W. H., Luppens, S. B. I., Marcelis, C. L. M. and Stams, A. J. M. (1998). Kinetics of acetate oxidation by two sulfate reducers isolated from anaerobic granular sludge. Appl. Environ. Microbiol. **64**: 2301-2303.

Oude Elfernik, S. T. W. H., Visser, A., Hulshoff Pol, W. and Stams, A. J. M. (1994). Sulfate reduction in methanogenic bioreactor. FEMS Microbiol. Rev. **15**: 119-136.

Paalme, T., Elken, R., Kahru, A., Vanatalu, K. and Vilu, R. (1997). The growth rate control in *Escherichia coli* at near to maximum growth rates: the A-stat approach. Antonie van Leeuwenhoek, **71**: 217-230.

Paalme, T., Kahru, A., Elken, R., Vanatalu, K., Tiisma, K. and Vilu, R. (1995). The computer-controlled continuous culture of *Escherichia coli* with smooth change of dilution rate (A-stat). J. Microbiol. Methods, **24**: 145-153.

Paller, G., Hommel, R. K. and Kleber, H-P. (1995). Phenol degradation by *Acinetobacter calcoaceticus* NCIB 8250. J. Basic. Microbiol. **35**: 325-335.

Parales, R. E., Ditty, J. L. and Harwood, C. S. (2000). Toluene-degrading bacteria are chemotactic towards the environmental pollutants benzene, toluene, and trichloroethylene. Appl. Environment. Microbiol. **66**: 4098-4104.

Pawlowsky, U. and Howell, J. A. (1973). Mixed culture biooxidation of phenol. I.. Determination of kinetic parameters. Biotechnol. Bioeng. **15**: 889-896.

Paxeus, N. P., Robinson, Balmer, P. (1992). Study of organic pollutants in municipal wastewater in Gotenberg, Sweden. Water Sci. Technol. **25**: 249-256.

Pirt, S. J. (1965). The maintenance energy of bacteria in growing cultures. Proc. R. Soc. London Ser. B. **163**: 224-231.

Rast, H. G., Engelhardt, G., Wallnofer, P. R., Oehlmann, L. and Wagner, K. (1979). Bacterial metabolism of substituted phenols: oxidation of 3-methyl-4(methylthio) phenol by *Nocardia sp*. DSM 43251. J. Agric. Food Chem. **27**: 699-702.

Readon, K. F., Mosteller, D. C. and Rogers, J. D. (2000). Biodegradation kinetics of benzene, toluene and phenol and mixed substrates for *Pseudomonas putida* F1. Biotechnol. Bioeng. **69**: 385-400.

Reber, H. H. and Kaiser, P. (1981). Regulation of the utilization of glucose and aromatic substrates in four strains of *Pseudomonas putida*. Arch. Microbiol. **130**: 243-247.

Reuss, M. and Fischer, P. (1991). Die zweistufige kontinuierliche Rührkesselkaskade-ein leistungsfähiges Handwerkszeug zum Studium des mikrobiellen Schadstoffabbaus bei Substratüberschußhemmung. gwf Wasser. Abwasser, **132**: 423-424.

Ribbons, D. W. (1970). Specificity of monohydric phenol oxidations by *meta* cleavage pathways in *pseudomonas aeruginosa* T1. Arch. Microbiol. **74**: 102-115.

Ribbons, D. W. and Eaton, R. W. (1982). Chemical transformations of aromatic hydrocarbons that support the growth of microorganisms. biodegradation and detoxification of environmental pollutants. CRC press, Boca Raton, Fla. p. 59-84.

Rizzuti, L., Augugliaro, V., Torregrossa, V. and Savarino, A. (1979). Kinetics of phenol removal by *Nocardia species*. Europ. J. Appl. Microbiol. Biotechnol. **8**: 113-118.

Roels, J. A. (1980). Application of macroscopic principles to microbial metabolism. Biotechnol. Bioeng. **22**: 2457-2514.

Rogers, J. B. and Readon, K. F. (2000). Modelling substrate interactions during the biodegradation of mixtures of toluene and phenol by *Burkholderia species* JS150. Biotech. Bioeng. **70**: 428-435.

Rozich, A. F. and Colvin, R. J. (1986). Effects of glucose on phenol biodegradation by heterogeneous populations. Biotechnol. Bioeng. **28**: 965-971.

Rubin, H. E. and Alexander, M. (1983). Effects of nutrients on the rates of mineralization of trace concentrations of phenol and p-nitrophenol. Environ. Sci. & Technol. **17**: 104-107.

Saéz, P. B. and Rittmann, B. E. (1993). Biodegradation kinetics of a mixture containing a primary substrate (phenol) and an inhibitory co-metabolite (4-chlorophenol). Biodegradation, **4**: 3-21.

Sala-Trepat, J. M. and Evans, W. C. (1971). The meta cleavage of catechol by *Azotobacter species*. 4-oxalocroconate pathway. Eur. J. Biochem. **20**: 400-413.

Salmond, C. V., Kroll, R. G. and Booth, I. R. (1984). The effect of food preservatives on pH homeostasis in *Escherichia coli*. J. Gen. Microbiol. **130**: 2845-2850.

Schmidt, S. K. and Alexander, M. (1985). Effects of dissolved organic carbon and second sustrates on the biodegradation of organic compounds at low concentrations. Appl. Environ. Microbiol. **49**: 822-827.

Schimp, R. J. and Pfaender, F. K. (1985). Influence of easily degradable naturally occuring carbon substrates on biodegradation of monosubstituted phenols by aquatic bacteria. Appl. Environ. Microbiol. **49**: 394- 401.

Schlegel, H. G. (1992). Allgemeine Mikrobiologie. 7 th edition. Georg Thieme Verlag, Stuttgart.

Schröder, M., Müller, C., Posten, C., Deckwer, W. -D. And Hecht, V. (1997). Inhibition kinetics of phenol degradation from unstable steady-state data. Biotechnol. Bioeng. **54**: 567-576.

Segel, I. H. (1975). Enzyme kinetics. New York, John Wiley & Sons.

Şeker, Ş., Beyenal, H. and Tanylaç, A. (1997). Multi-substrate growth kinetics of *Pseudomonas putida* for phenol removal. Appl. Microbiol. Biotechnol. **47**: 610-614.

Senn, H., Lendenmann, U., Snozzi, M., Hamer, G. and Egli, T. (1994). The growth of *Escherichia coli* in glucose-limited chemostat cultures: a re-examination of the kinetics. Biochim. Biophys. Acta, **1201**: 424-436.

Sheela, S. and Pai, S. B. (1983). Metabolism of fensulfothion by a soil bacterium, *Pseudomonas alcaligenes* C_1. Appl. Environ. Microbiol. **46**: 475-479.

Shields, M. S., Montgomery, S. O., Chapman, P. J., Cuskey, S. M., and Pritchard, P. H. (1989). Novel pathway of toluene catabolism in the trichloroethylend-degrading bacterium G4. Appl. Environ. Microbiol. **55**: 1624-1629.

Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J. and Pritchard, P. H. (1991). Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environment. Microbiol. **57**: 1935-1941.

Shim, H. and Yang, S.-T. (1999). Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* immobilized in a fibrous-bed bioreactor. J. Biotechnol. **67**: 99-112.

Singirtsev, I. N., Volchenko, E. V., Korzhenevich, V. I., Gumenyuk, A. P. and Fedorov, A. Y. (2000). Microbial degradation of components of sewage from phenol production facilities. Appl. Biochem. Microbiol. **36**: 150-159.

Singleton, I. (1994). Microbial metabolism of xenobiotics: fundamental and applied research. J. Chem. Tech. Biotechnol. **59**: 9-23.

Sipkema, E. M., de Koning, W., Ganzeveld, K. J., Janssen, D. B. and Beenackers, A. A. C. M. (1998). Experimental pulse technique for the study of microbial kinetics in continuous culture. J. Biotechnol. **64**: 159-176.

Smith, M. R. (1990). The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation, **1**: 191-206.

Smith, M. R., Erwing, M. and Ratledge. C. (1991). The interactions of various aromatic substrates degraded by *Pseudomonas sp*. NCIB 10643: synergistic inhibition of growth by two compounds that serve as growth substrates. Appl. Microbiol. Biotechnol. **34**: 536-538.

Sokoł, W. (1987). Oxidation of an inhibitory substrate by washed cells (oxidation of phenol by *Pseudomonas putida*). Biotechnol. Bioeng. **30**: 921-927.

Sokoł, W. (1988a). Dynamics of continuous stirred-tank biochemical reactor utilizing inhibitory substrate. Biotechnol. Bioeng. **31**: 198-202.

Sokoł, W. (1988b). Uptake rate of phenol by *Pseudomonas putida* grown in unsteady state. Biotechnol. Bioeng. **32**: 1097-1103.

Sokoł, W. and Howell, J. A. (1981). Kinetics of phenol oxidation by washed cells. Biotechnol. Bioeng. **23**: 2039-2049.

Solomon, B. O., Posten, C., Harder, M. P. F., Hecht, V. and Deckwer, W.-D. (1994). Energetics of *Pseudomonas cepacia* G4 growth in a chemostat with phenol limitation. J. Chem. Tech. Biotechnol. **60**: 275-282.

Spain, J. C. and Nishino, S. F. (1987). Degradation of 1,4-dichlorobenzene by a *Pseudomonas sp*. Appl. Environ. Microbiol. **53**: 1010-1019.

Spånning, Å. and Neujahr, H. Y. (1987). Growth and enzyme synthesis during continuous culture of *Trichosporon cutaneum* on phenol. Biotechnol. Bioeng. **29**: 464-468.

Stanier, R. Y. and Ornston, L. N. (1973). The ß-ketoadipate pathway. Adv. Microbiol. Physiol. **9**: 89-151.

Stainer, R. Y., Sleeper, B. P., Tsushida, M. and McDonald, D. L. (1950). Bacterial oxidation of aromatic compounds. III. The enzymatic oxidation of catechol and protocatechuic acid to ß-ketoadipate acid. J. Bacteriol. **53**: 137-151.

Straube, G. (1987). Phenol hydroxylase from *Rhodococcus sp*. P1. J. Basic Microbiol. **27**: 229-232.

Tan, Y., Wang, Z.-X. and Marshall, K. C. (1996). Modelling substrate inhibition of microbial growth. Biotechnol. Bioeng. **52**: 602-608.

Timmis, K. N., Rojo, F. and Ramos, J. L. (1988). Prospects for laboratory engineering of bacteria to degrade pollutants. Basic. life sci. **45**: 61-79.

van der Sluis, C., Westerink, B. H., Dijkstal, M. M., Castelein, S. J., Van Boxtel, A. J. B., Giuseppin, M. L. F., Tramper, J. and Wijffels, R. H. (2001). Estimation of steady-state culture characteristics during acceleration-states with yeasts. Biotech. Bioeng. **75** (3): 267-275.

van der Woude, B. J., Gottschal, J. C. and Prins, R. A. (1995). Degradation of 2,5-dichlorobenzoic acid by Pseudomonas aeruginosa JB2 at low oxygen tensions. Biodegradation, **6**: 39-46.

Villarejo, M., Stanovich, J., Young, K. and Edlin, G. (1978). Differences in membrane proteins, cyclic AMP levels, and glucose transport between batch and chemostat cultures of *Escherichia coli*. Current Microbiol. **1**: 345-348.

Vojta, V., Náhlík, J., Páca, J. and Komárková, E. (2002). Development and verification of the control system for fed-batch phenol degradation processes. Chem. Biochem. Eng. Q. **16**: 59-67.

Wang, K.-W., Baltzis, B. C. and Lewandowski, G. A. (1996). Kinetics of phenol biodegradation in the presence of glucose. Biotech. Bioeng. **51**: 87-94.

Wang, S.-J. and Loh, K.-C. (1999). Modelling the role of metabolic intermediate in kinetics of phenol biodegradation. Enzyme and Microbial Technology, **25**: 177-184.

Wang, S.-J. and Loh, K.-C. (2000). Growth kinetics of *Pseudomonas putida* in cometabolism of phenol and 4-chlorophenol in the presence of a conventional carbon source. Biotechnol. Bioeng. **68**: 437-447.

Wang, S.-J. and Loh, K.-C. (2001). Biotransformation kinetics of *Pseudomonas putida* for cometabolism of phenol and 4-chlorophenol in the presence of sodium glutamate. Biodegradation, **12**: 189-199.

Wang, Y.-S., Madsen, E. L. and Alexander, M. (1985). Microbial degradation by mineralization or cometabolism determined by chemical concentration and environment. J. Agric. Food Chem. **33**: 495-499.

Wayman, M. and Tseng, M. C. (1976). Inhibition-threshold substrate concentrations. Biotechnol. Bioeng. **18**: 383-387.

Weitzman, P. D. J. and Jones, D. (1968). Regulation of citrate synthase and microbial taxonamy. Nature (Londen), **219**: 270-272.

Williams, P. A. and Sayer, J. R. (1994). The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation, **5**: 195-217.

Wright, J. D. and Ratledge, C. (1991). Isolation of two *Rhodotorula rubra* strains showing differences in the degradation of aromatic compounds. Appl. Microbiol. Biotechnol. **35**: 94-99.

Yamaguchi, M. and Fujisawa, H. (1980). Purification and characterization of an oxygenase component in benzoate 1,2-dioxygenase system from *Pseudomonas arvilla* C-1. J. Biol. Chem. **255**: 5058-5063.

Yamaguchi, M. and Fujisawa, H. (1982). Subunit structure of oxygenase component in benzoate-1,2-dioxygenase system from *Pseudomonas arvilla* C-1. J. Biolog. Chem. **257**: 12497-12502.

Yang, R. D. and Humphrey, A. E. (1975). Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures. Biotechnol. Bioeng. **17**: 1211-1235.

Yano, T. and Koga, S. (1969). Dynamic behaviour of the chemostat subject to substrate inhibition. Biotechnol. Bioeng. **11**: 139-153.

Zeyer, J. and Kearney, P. C. (1984). Degradation of o-nitrophenol and m-nitrophenol by a *Pseudomonas putida*. J. Agric. Food Chem. **32**: 238-241.

Curriculum Vitae

Personal data:

Name:	Moustafa El-Sayed Abd El-Hameid Shalaby
Date of birth:	25 October, 1966
Place of birth:	Kafr El-Sheikh, Egypt
Nationality:	Egyptian
Marital status:	Married and have two children, Nourhan and Ahmed

Academic qualifications:

1972 - 1978	Primary school, Kafr El-Sheikh, Egypt
1978 - 1981	Preparatory school, Kafr El-Sheikh, Egypt
1981 - 1984	Secondary school, Kafr El-Sheikh, Egypt
1984 - 1988	Faculty of Agriculture, Kafr El-Sheikh, Tanta University, Egypt
1988	B. Sc.: Agricultural Sciences (Agricultural Botany Dept.), Kafr El-Sheikh Faculty of Agriculture, Tanta University, Egypt
1995	M. Sc.: Agricultural Sciences (Agricultural Botany Dept.), Kafr El-Sheikh Faculty of Agriculture, Tanta University, Egypt Title of M. Sc. Thesis: "Studies on plant life at Kafr El-Sheikh province,Egypt".

Professional employments:

1989 - 1995	Demonstrator, Agricultural Botany Department, Kafr El-Sheikh Faculty of Agriculture, Tanta University, Egypt
1995 - 1999	Assistant Lecturer, Agricultural Botany Department, Kafr El-Sheikh Faculty of Agriculture, Tanta University, Egypt
1999 - till now	Ph. D. researcher (student) in the Biochemical Engineering Division of the GBF (Gesellschaft für Biotechnologische Forschung mbH), Mascheroder Weg 1, D-38124 Braunschweig, Germany Title of Ph. D. Thesis: "Biological degradation of substrate mixtures composed of phenol, benzoate and acetate by *Burkholderia ceoacia* G4".